THE EPIDEMIOLOGY OF BOVINE TUBERCULOSIS (*Mycobacterium bovis*) IN THE GREATER RIDING MOUNTAIN ECOSYSTEM

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By

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ABSTRACT

The overall objective of this thesis is to provide an enhanced understanding of the epidemiology of *Mycobacterium bovis* in the Greater Riding Mountain Ecosystem (GRME) and provide a scientific basis for disease management from a systems perspective now and into the future. *M. bovis* prevalence has been consistently higher in elk compared to white-tailed deer, and higher within a defined Core area compared to areas outside. Prevalence in both species declined significantly between 2003 and 2013. Only one infected elk was detected in 2013; the last infected white-tailed deer was detected in 2009 and the last infected cattle herd was detected in 2008. Parallel interpretation of three blood-based assays resulted in effective selective culling of elk within Riding Mountain National Park (RMNP) with predictive value negative of 100%. A lymphocyte stimulation test (LST) was the most sensitive single blood-based assay, but was difficult to perform under field conditions. Combinations of humoral antibody tests and cell-mediated tests performed better than any single test, likely detecting the broad spectrum of host pathology present. Seven of 14 risk factors were identified for wild cervids testing culture positive with the three being most strongly associated with culture positivity being geographical location (within core area), elk density and year category (sampling phase). Age, sex, and surveillance method were also significant factors, but species was not. A rapid decline in elk density in combination with fencing of hay storage yard and non-selective culling were likely key factors resulting in the *M. bovis* prevalence decline observed in elk, and an overall decline in prevalence from 1997 for both species. Elk were the primary reservoir species in this episystem, but are now considered a spillover host, while white-tailed deer have always been a spillover host due to lower densities and shorter life expectancy. Very limited strain diversity exists within

the GRME with one spoligotype restricted to cattle and associated with a limited outbreak in five herds in the early 1990's, and three other shared strains between cattle and wildlife. A single monomorphic type was present in white-tailed deer. Significant spatial overlap of wildlife and cattle isolates delineated a core area where management activities are now focused. The relative simplicity of this episystem has allowed significant progress on control and management to be achieved, despite being located within a national park. Wildlife surveillance will need to continue until at least 2022 in order to achieve a 95% probability of freedom using three different surveillance streams. Latent cases are likely to be extremely rare in future and unlikely to result in ongoing transmission as the factors that created this wildlife reservoir no longer exist. Wild cervids should not be considered ideal maintenance hosts for *M. bovis* in North America but rather facultative hosts; acting either as a reservoir or spillover host dependent on regional/local density and presence/absence of baiting and feeding.

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DEDICATION

This thesis is dedicated to my parents, Garry and Claudia Shury who somehow managed to instill a sense of curiosity about the natural world in their youngest son and allowed me the freedom to explore that world when the time came.

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LIST OF ABBREVIATIONS

AUC – AREA UNDER THE CURVE
CFIA – CANADIAN FOOD INSPECTION AGENCY
DMPFF – DUCK MOUNTAIN PROVINCIAL PARK & FOREST
DR – DIRECT REPEAT REGION
EPI – EFFECTIVE PROBABILITY OF INFECTION
FPA – FLOURESCENCE POLARIZATION ASSAY
GRME – GREATER RIDING MOUNTAIN ECOSYSTEM
GVL – GROSS VISIBLE LESIONS
LST – LYMPHOCYTE STIMULATION ASSAY
MDCE – MYCOBACTERIAL DISEASES CENTRE OF EXCELLENCE
MIRU – MYCOBACTERIAL INTERSPERSED REPETITIVE UNITS
MLVA – MULTI LOCUS VARIABLE NUMBER OF TANDEM REPEAT ANALYSIS
MTC – *MYCOBACTERIUM TUBERCULOSIS* COMPLEX
OTF – OFFICIALLY TUBERCULOSIS FREE
PCR – POLYMERASE CHAIN REACTION
PPD – PURIFIED PROTEIN DERIVATIVE
RFLP – RESTRICTION FRAGMENT LENGTH POLYMORPHISM
RM – RURAL MUNICIPALITY
RMEA – RIDING MOUNTAIN ERADICATION AREA
RMNP – RIDING MOUNTAIN NATIONAL PARK
ROC – RECEIVER OPERATING CURVE
SRC – SCIENTIFIC REVIEW COMMITTEE
VNTR – VARIABLE NUMBER OF TANDEM REPEATS
WTD – WHITE-TAILED DEER
NVL – NO VISIBLE LESIONS

CHAPTER 1: OVERVIEW AND BACKGROUND OF WILDLIFE RESERVOIRS OF *MYCOBACTERIUM BOVIS*

1.1 Global overview: the organism and its hosts

The *Mycobacterium tuberculosis* complex (MTC) is a group of very closely related mycobacterial pathogens with a large host range that include humans, wild and domestic terrestrial and marine mammals, birds and reptiles. This complex includes *M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*, *M. caprae*, *M. pinnipedii*, *M. cannetti*, and several non-pathogenic or opportunistic mycobacteria (Mostowy et al., 2005; Forrellad et al., 2013). Approximately 1/3 of the global human population (2 billion) are currently infected with *M. tuberculosis* and there were 8.6 million incident cases in 2012 (World HealthOrganization, 2013). While a much smaller proportion (1.4% to 3.1%) of the global human population are infected with zoonotic *M. bovis*, the organism typically associated with domestic cattle and wildlife, the incidence of *M. bovis* is difficult to accurately estimate in many countries and the incidence is likely vastly under-reported (Muller et al., 2013; Perez-Lago et al., 2014). *M. bovis* includes one of the broadest host ranges of any pathogen discovered to date (Table 1) and shares 99.9% nucleotide homology with other members of the MTC (Smith, 2012). Both *M. bovis* and *M. tuberculosis* evolved from a common ancestor 10,000 to 20,000 years ago and have evolved into ecotypes specializing in infecting cattle and humans respectively, and both have evolved over time by losing segments of their genome (regions of difference RD)(Brosch, 2002; Smith et al., 2006b; Michel et al., 2010). The first tuberculosis vaccine developed for use in humans was an attenuated form of *M. bovis* known as BCG (Bacille-Calmette-Guerin), and it is still in wide use for vaccination in children in high prevalence settings.

M. bovis is an aerobic, gram-positive, slow-growing, acid-fast bacterium that possesses a unique lipid-rich cell wall containing peptidoglygans and glycolipids, which allows it to evade

the host immune response within granulomata and remain latent for long periods of time, including the lifespan of the host (Kaneene and Thoen, 2004; Gengenbacher and Kaufmann, 2012). Transmission in both cattle and cervids occurs primarily in natural settings through aerosol or droplet spread, but also indirectly through shared feed and occasionally via vertical or pseudo-vertical transmission through milk (Morris et al., 1994; O'Reilly and Daborn, 1995; Palmer et al., 2012). Visible lesions are typically granulomatous and often mineralized in cattle, while in cervid species they are most often caseogranulomas with purulent centres (Fitzgerald and Kaneene, 2013). Lesions in both wild and domestic cervid species primarily occur in the lungs or associated thoracic lymph nodes, or in the lymph nodes of the head, specifically the medial retrophyngeal lymph node (RPLN) (de Lisle et al., 2002; O'Brien et al., 2002; O'Brien et al., 2008; Fitzgerald and Kaneene, 2013). In both white-tailed deer (*Odocoileus virginianus*) and red deer (*Cervus elaphus*), the RPLN has been the most common anatomic site where grossly visible lesions occur and the most sensitive in terms of surveillance (Lugton et al., 1997; Fitzgerald et al., 2000; Palmer et al., 2002) In other wildlife reservoir species such as ferrets (*Mustela putorius furo*) and brush-tailed possum (*Trichosurus vulpecula*), transmission can occur through bite wounds or draining fistulous tracts (Fitzgerald and Kaneene, 2013) and lesions are generally found in more diverse sites.

M. bovis has severe socioeconomic consequences for cattle producers and government agencies in some countries; the Michigan Dept. of Natural Resources expended approximately US$23 million to 2011 (O'Brien et al., 2011a), while in Great Britain bTB control cost approximately £100 million annually (McDonald, 2014). This results in ongoing zoonotic infections in humans in many parts of the world where pasteurization of milk products is not as common as in the developed world. It occurs primarily in foreign-born persons in most

industrialized countries, including Canada that practice widespread pasteurization of milk (Long et al., 1999; Varughese et al., 2014). *M. bovis* represents a barrier to trade in many industrialized nations which have spent millions of dollars eliminating bovine tuberculosis (bTB) from their national cattle herds, mainly to benefit from increased trade in cattle and their products without expensive export testing regimes (Kaneene and Thoen, 2004; Koller-Jones et al., 2006).

A wildlife reservoir constitutes one or more epidemiologically connected populations in which *M. bovis* can be permanently maintained and is subsequently transmitted to various target populations which may be reservoir or spillover hosts (Haydon et al., 2002; Palmer, 2013). It is important to note that one or more species of host may be involved in creating and maintaining a wildlife reservoir, but sorting out the different status of host species is often difficult and takes a long time to ascertain properly. Well known wildlife reservoirs of *M. bovis* include the brush-tailed possum (New Zealand), red and fallow deer (*Dama dama*, Spain/Portugal), bison (*Bison bison athabascae*, Canada), wild boar (*Sus scrofa*, Spain/Portugal), European badger (*Meles meles*, United Kingdom, Ireland), African buffalo (*Syncercus caffer*, South Aftrica, Mozambique), and white-tailed deer (USA) (Table1). Some characteristics of these species lend themselves well to being reservoir hosts for *M. bovis* (highly social, susceptible to *M. bovis*, occur as relatively dense host populations), while with other species (white-tailed deer and wild boar) their host status depends more on their ecological and epidemiological situation (host density, presence of congregating factors)(Palmer, 2013).

In Australia, feral hogs were considered a spillover host for *M. bovis* due to lesion localization in peripheral lymph nodes and lack of generalized bTB, and so during eradication this species was essentially ignored in the northern Territories with no control and the infection disappeared once it was controlled in the primary reservoir species, feral water buffalo (*Bubalus*

arnee) and cattle (Corner et al., 1981; Radunz, 2006). Similarly, in New Zealand where wild boar are considered good sentinels for the presence of *M. bovis* in a region, due to their status as a spillover host in which infection develops relatively quickly, surveillance activities are less expensive than it is in the primary reservoir host (brush-tailed possums). Conversely, wild boar in Spain and Portugal exist at relatively high densities, are artificially fed to increase hunting opportunities on estates, congregate at water holes with other reservoir species and have high prevalence and rates of generalized lesions (Vicente et al., 2007b; Gortazar et al., 2008; Naranjo et al., 2008). In this context, wild boar are considered reservoir hosts rather than spillover hosts due to ecological circumstances which facilitate intraspecific transmission and maintenance of *M. bovis* above a critical community size (CCS).

Evidence from New Zealand, both empirical and model-based, indicates that reduction of brush-tailed possum densities below a critical threshold (Caley and Hone, 2005; O'Brien et al., 2011b) for a prolonged period of time, causes *M. bovis* to become eliminated from both possum populations as well as other epidemiologically connected populations (ferrets, red deer, wild boar, cattle). This is the basis of the New Zealand national control program which has been highly effective in reducing prevalence in cattle and farmed deer as well as the overall geographical extent of infection in the country (Morris et al., 1994; O'Brien et al., 2011b; Fitzgerald and Kaneene, 2013).

1.2 Bovine tuberculosis eradication

Attempts to control bTB in domestic animal populations are severely hampered when a wildlife reservoir exists, due to problems with spillback from infected wildlife. Eradication of bTB in domestic bovids is well defined by the World Organization for Animal Health (OIE) as the point when *"…regular and periodic testing of all cattle, water buffalo and wood bison herds demonstrated that M. bovis infection was not present in at least 99.8 percent of the herds and*

99.9 percent of the cattle, water buffaloes and wood bisons in the country or zone for three consecutive years." (World Organization for Animal Health, 2014). Countries that wish to declare themselves free of bTB typically achieve eradication through intensive live animal testing and abattoir surveillance of slaughtered bovines. There are no international standards for country or zone freedom from *M. bovis* in wildlife populations, and so countries must use domestic animal guidelines and adapt them to wildlife populations where necessary. Consequently, elimination is a more appropriate term for control of *M. bovis* in wildlife reservoirs as defined by Dowdle (1998), as the "*Reduction to zero of the incidence of infection caused by a specific agent in a defined geographical area as a result of deliberate efforts; continued measures to prevent re-establishment of transmission are required*." Control measures that have been attempted for *M. bovis* in wildlife reservoirs including aerial poisoning, density reduction, culling, and vaccination.

There are few examples where these control measures would be considered empirically 'successful'. One of the few examples of success is Australia where national bTB eradication program has been in place since 1970 (Cousins and Roberts, 2001). A major aspect of the Australian campaign was the destocking (depopulation) of feral cattle and water buffalo from the northern territories by aerial shooting using helicopter marksman (Cousins and Roberts, 2001). New Zealand has relied primarily on aerially applied 1080 to reduce brush-tailed possum density as a control measure, while culling European badgers has been very controversial in the United Kingdom, but has been used in the Republic of Ireland as a management tool with some apparent success (O'Connor et al., 2012).

Infected cattle farms were discovered in the northwest portion of Minnesota in 2007 and white-tailed deer were found in close proximity to case farms, prompting a rapid response which

involved increased hunting opportunities for deer as well as ground and aerial sharpshooting to reduce deer density in the affected counties, in concert with buyout of cattle farms in a defined core area (a 1,567 km^2 area delineated by a 16 km radius around 7 index *M. bovis* positive WTD) (Carstensen and Doncarlos, 2011; Carstensen et al., 2011). This is one of the few areas where *M. bovis* appears to have been eliminated from a 'potential' wildlife reservoir, likely due to rapid, intense management and low deer density present at the beginning of the outbreak. This is in contrast to the situation in Michigan, where *M. bovis* prevalence dropped initially in response to deer density reduction, but stakeholder acceptance issues prevented rapid control and management of deer baiting and feeding issues resulting in a stable endemicity in free-ranging white-tailed deer (O'Brien et al., 2006; 2011a).

1.3 Wildlife reservoirs of *M. bovis* in Canada

There are two known wildlife reservoirs of *M. bovis* in wildlife in Canada, both within national parks. The one with the longest history is found in wood bison in and around Wood Buffalo National Park in northern Alberta and the Northwest Territories. This population of bison which is currently estimated to number approximately 5,000 animals is co-infected with *Brucella abortus* and infection with these agents likely resulted from the translocation of 6,673 infected plains bison (*Bison bison bison*) from Buffalo National Park between 1925 and 1928. *Mycobacterium bovis* has never spilled over into domestic animal populations from this wildlife reservoir to date, but the potential for this occur exists, despite the relative isolation of this bison population from domestic cattle.

Sporadic occurrences of *M. bovis* in wild cervids has been documented in Canada, but the only place where this has resulted in a wildlife reservoir of *M. bovis* has been the area around Riding Mountain National Park. Belli (1962), found a single case of *M. bovis* in a white-tailed deer (sex unreported) near Gravenhurst, Ontario, within a sample of 440 hunter-killed deer (0.2%

prevalence) in 1958, and *M. bovis* has never been reported from wild cervids in Ontario since that time, despite intensive surveillance for chronic wasting disease (CWD) that has occurred in the last decade. The only other report of *M. bovis* in wild cervids was in association with infected plains bison in Buffalo National Park where Hadwen (1942) found *M. bovis* in 0.8% (n=242) of mule deer (*Odocoileus hemionus*), 5.6% (n= 107) of moose (*Alces alces*), 5.5% (n = 1329) of elk (*Cervus canadensis*) and 53.7% of bison (*Bison bison bison*) (n = 6,450). This situation was exceptional, as all of the wild ungulates within the fenced area of Buffalo National Park were eradicated in 1939 due to the high levels of infection found in the plains bison. It is likely that this action prevented the establishment of a wildlife reservoir in this location. This also illustrates that high levels of infection can be found in wild cervids when they occur at high densities with a source of infection and transmission (i.e. bison) closely associated.

M. bovis has also been diagnosed in captive cervids in Canada, with a major outbreak in the early 1990's in Alberta associated with imported elk from the US and sporadic cases being found since then (Koller and Essey, 1994; Wobeser, 2009).

The discovery of an infected male elk in the Rural Municipality (RM) of Rossburn south of Riding Mountain National Park in October 1992 was the first documented case of *M. bovis* in a wild elk in North America. This was a hunter-killed elk, and at the time was thought to be an isolated case, and not a wildlife reservoir. It was not until September of 1998 when another *M. bovis* infected male elk was found dead within Riding Mountain National Park, that it was considered that there might be a wildlife reservoir in this area. The only other documented case of *M. bovis* in wildlife in this area prior to 1992 was a case report of two gray wolves (*Canis lupus*) found dead in Riding Mountain National Park in 1978 (Carbyn, 1982). These animals were both wolf pups (< 1 year of age) likely from same litter. They may also have been co-

infected with canine distemper which could have predisposed them to other infectious diseases such as bovine tuberculosis, as several other wolves were found dead at the same time from distemper (Carbyn, 1982). Formalinized tissues containing *M. bovis* from one of these wolves were later spoligotyped and found to be the same strain type found in elk, white-tailed deer and cattle (MB-1, SB1070) in this area since 1992 (Lutze-Wallace et al., 2005a), indicating that this strain type has been circulating in this ecosystem since at least 1978.

Remarkably, 98 elk translocated from within Riding Mountain National Park (RMNP) between 1968 and 1971 to the Interlake region of Manitoba were disease tested using tuberculin skin injection and found negative for both tuberculosis and brucellosis (Brook, 2007). The population of elk resulting from this translocation is considered to be free of both diseases, although limited or no testing has occurred since they were translocated (personal communication Richard Davis, Manitoba Conservation). In addition, 175 elk were captured to be sold into the domestic cervid industry in Manitoba near the Duck Mountains and near the boundary of RMNP in the winter of 1998/99. Because these animals were destined to be source stock for the fledgling Manitoba captive cervid industry, 166 of the founder animals captured were subjected to mid-cervical tuberculin tests; 27 reactors were found, but all were culture negative following slaughter and examination at necropsy. The progeny of these animals were also subjected to 2 mid-cervical tuberculin tests with 11 reactors being found, but all 11 were negative on culture after necropsy. The remaining 135 founder animals were all subjected to euthanasia and examination after having calves and found to be culture negative for *M. bovis*, although 8 animals were cultured positive for other mycobacteria including *M. terrae* and *M. avium*. This would indicate that if *M. bovis* was present in the elk population during the 1970's

and late 1990's, it was at a very low prevalence, was a very recent introduction or was not widely distributed in elk populations.

The origin of *M. bovis* in this wildlife reservoir is unknown, but it is likely from one of two sources. The most likely possibility of the origin in wildlife is from cattle which were grazed within RMNP until 1970 during summer grazing seasons. Bovine tuberculosis was not uncommon in cattle during the 1950's and 1960's while Manitoba was undergoing eradication of both tuberculosis and brucellosis in the domestic cattle herd, and there is anecdotal evidence that elk in the area of Riding Mountain National Park were infected with tuberculosis (Brook, 2007; Brook, 2009).

The second possibility is that *M. bovis* was transmitted from infected plains bison which were held in a display pen from starting in 1931. These bison were held in a 1.4 square kilometer pen near Lake Audy and all 20 bison (4 bulls, 16 cows) were shipped from Buffalo National Park near Wainwright, Alberta, a herd known at the time to be heavily infected with bovine tuberculosis (Zhao, 2006). Bovine tuberculosis was confirmed in these bison in 1937 and at least one release of elk which were co-housed with these infected bison occurred in 1937 and likely occurred subsequent to this as well (Zhao, 2006). The original bison herd was subsequently slaughtered in 1947 and replaced with disease-free plains bison from Elk Island National Park, but the elk that inhabited the enclosure with the infected bison were simply released into the park. Zhao concluded that the source of infection with *M. bovis* was from the infected bison from Buffalo National Park. While this is a possibility, it seems less likely than a spillover from possibly infected cattle which were grazed in the park. One reason for this is that only two closely related spoligotypes of *M. bovis* have been detected since 1991 in elk, deer and cattle in the area of southeastern Manitoba (Lutze-Wallace et al., 2005a; Lutze-Wallace et al., 2005b) and

these differ substantially from the only spoligotype that has been found in Wood Buffalo National Park (Lutze-Wallace et al., 2006), which was also infected by translocated bison from Buffalo National Park during the 1920's (Pybus and Shury, 2012). While it is possible that an undetected spoligotype existed within the Buffalo National Park bison herd that was translocated to RMNP in 1931 along with the infected bison, it is very unlikely that this spoligotype has never been detected since. The strain of *M. bovis* found within the GRME is part of the EU1 clonal complex (Smith, 2012) which is the most common clonal complex worldwide, so we can say with some certainty that the infection originally came from cattle and is not an 'ancestral' strain of *M. bovis* that has been present in wildlife, but not cattle.

Preliminary contingency table analyses and reports from wildlife surveillance in the GRME indicated that male elk (5% prevalence) were more likely to be infected than female elk, that prevalence increased with age in elk and that the overall prevalence of infection was approximately 1% in elk (Lees et al., 2003; Lees, 2004). These studies also suggested two foci of infection within the GRME, one in the west side of RMNP including the municipalities of Rossburn and Grandview and another in the municipality of Park South (Lees et al., 2003). Other studies revealed information about farmer attitudes towards bovine tuberculosis and effectiveness of barrier hay fencing for reducing contact between wildlife and cattle (Brook and McLachlan, 2006; Brook, 2010; Gooding and Brook, 2014).

Coyotes from the GRME were sampled in 2004/2005 to determine infection rates in this species using culture, PCR and histopathology of retropharyngeal, colonic, and mesenteric lymph nodes and tonsils, with no infected individuals were found in a sample of 82 coyotes from municipalities bordering RMNP (Sangster et al., 2007), indicating that coyotes are not good sentinels in this ecosystem where wolves are the top canine predator. Wolves are also not good

sentinels of *M. bovis* infection in the GRMEA, as multiple tissues from 136 wolves have been examined for presence of tuberculous lesions by culture and presence of grossly visible lesions between 1992 and 2012 in the Riding Mountain Eradication Area (RMEA), all with negative findings to date (unpublished data Parks Canada Agency). In contrast, data from the US state of Michigan indicate that coyotes make very useful sentinels of *M. bovis* infection, due to relatively high prevalence (4.8% to 30%) and lack of overt clinical infections (Bruning-Fann et al., 2001; Atwood et al., 2007; VerCauteren, 2008).

Riding Mountain National Park was created in 1930 sits on a large escarpment above the surrounding plains and is a southern extension of the boreal forest ecosystem in Canada. It has been described as an island of wilderness in a sea of agriculture, with agricultural lands surrounding the boundary of the park on all sides (Brook, 2009).

A substantial body of social and ecological research has been conducted on elk in the GRME, with some research on white-tailed deer ecology as well in the past decade (Brook and McLachlan, 2006; Brook, 2007; Brook, 2009; Brook and McLachlan, 2009; Brook, 2010; Vander Wall, 2011; Vander Wal et al., 2012a; Vander Wal et al., 2012b; Brook et al., 2013; Dugal et al., 2013; van Beest et al., 2013; Vander Wal et al., 2013a; Vander Wal et al., 2013b; Gooding and Brook, 2014; van Beest et al., 2014a; van Beest et al., 2014b). There is a long history of elk agriculture conflicts in this region since the late 19th century when intensive agriculture began on the Canadian prairies (Brook, 2009). It is clear that most cattle-elk interaction takes place within 6 kilometres of the boundary of RMNP and mostly during the spring and early summer seasons (Brook and McLachlan, 2009). The amount of forest cover present in cattle pastures was also strongly positively correlated, with elk use around RMNP with cattle and elk sharing pastures, water and mineral sources. White-tailed deer use of cattle winter

feeding areas around RMNP was higher than elk use and was influenced by cattle herd size, distance to RMNP boundary, amount of forest cover and whether or not round bale feeders were used (Brook et al., 2013). Local cattle producers also expressed serious concerns about *M. bovis* in wildlife populations within and around RMNP, as well as concerns about baiting and feeding of wildlife (Brook and McLachlan, 2006), but did not generally support removing the entire elk population (depopulation) or fencing the park as management solutions (Brook et al., 2013).

1.4 Management and surveillance of *M. bovis* in the Greater Riding Mountain Ecosystem (GRME)

The Greater Riding Mountain Ecosystem (GRME) comprises a rectangular portion of southwestern Manitoba along the Saskatchewan border (Figure 1.1). It encompasses two protected areas; Riding Mountain National Park in the southern half and the Duck Mountain Provincial Park and Forest in the northern half. Both protected areas are southern extensions of the Boreal Plains ecozone comprising part of the Mid-Boreal Uplands ecoregion which are elevated uplands along the Manitoba Escarpment above the surrounding plains which protect key populations of large ungulates such as elk, moose and deer as well as their associated predators (gray wolves, black bears)(Smith et al., 1998).

Bovine TB reactor prevalence in cattle using the caudal fold tuberculin test in the 15 rural municipalities surrounding RMNP varied between 0.11% and 3.25% between 1916 and 1953, with higher reactor prevalence being found in the eastern portion (RM's of McCreary, Rosedale, Ochre River and Ste. Rose)(Zhao, 2006). Manitoba became one single restricted area for bTB testing in 1951 and the province was declared free of bTB in 1986. Just five years later, a bTB positive slaughter cow was found, the source of which traced back to a herd in the RM of Rossburn on the south side of RMNP. The subsequent investigation tested 15,500 head of cattle from 250 farms (Pers. comm. L. Bates CFIA, Munroe et al 1999). Caudal fold reactor from 18

herds were found and 5 of these herds with 1,000 head of cattle had culture positive cattle at necropsy and were subsequently depopulated (all cattle on positive farms slaughtered) (Munroe et al., 1999). The index herd (herd A) was infected through sale of an infected animal from the RM of Rossburn that was pastured next to an infected herd (herd D). This source herd (D) was approximately one mile from an *M. bovis* positive bull elk that was subsequently killed by hunter in October 1992.

A small special hunt instituted in the winter of 1992/93 found no positive elk, white-tailed deer or moose from a sample of 55 animals. Another Manitoba slaughter cow was found to be *M. bovis* positive at a US slaughter plant in October of 1997. This animal traced back to a farm (herd E) in the RM of Rossburn, in close proximity to the source herd from the 1991 outbreak (herd D). This source herd (E) contained a high prevalence of culture positive cattle (30/85 head – 35.3%) and had tested negative to the caudal fold test when the entire herd was tested as part of the investigation in 1991. This herd was depopulated as was another infected herd (F), which was infected through sale of cattle from the source herd (E). Another 56 exposed or traced cattle herds (~2,500 head) were tested in the region as part of the outbreak investigation and all were found to be negative after caudal fold testing of live animals in these herds.

As local wildlife were now suspected of being potentially involved in this outbreak, a comprehensive cooperative wildlife health monitoring program began in the fall of 1997 which examined heads and lungs from 200 hunter-killed elk (n=139), white-tailed deer (n=6) and moose (n=55) from rural municipalities surrounding RMNP, all of which were culture negative. Interestingly, two elk were found with lesions at necropsy that were acid-fast positive, and subsequently *Mycobacterium avium* was cultured. This prompted local area testing of 55 cattle herds (2,479 head), all of which were negative on the caudal fold test. A bull elk was

subsequently found near Lake Audy in September 1998 which had been gored by another bull, but was *M. bovis* culture positive. Nine more *M. bovis* culture positive elk were found through hunter surveillance between 1998 and 2001, all within RMNP or within a few kilometers of the north or south boundary.

In response, the Manitoba Bovine Tuberculosis Management Program created a Bovine TB Implementation Plan to help manage the *M. bovis* outbreak which involved both domestic livestock and wildlife. This subsequently became the Manitoba Bovine TB Task Force, which developed annual Implementation Plans through cooperation of four government departments; the Canadian Food Inspection Agency (CFIA), Parks Canada Agency, Manitoba Conservation and Manitoba Agriculture, Food and Rural Initiatives (MAFRI).

The first *M. bovis* positive white-tailed deer was found in the RM of Rossburn in the winter of 2001, and a *M. bovis* positive cattle herd was identified in May of 2001in the RM of Grandview. This prompted the US Dept. of Agriculture (USDA) to put import restrictions on breeding cattle of Manitoba origin not going to direct to slaughter in the US. In response, the Canadian Food Inspection Agency (CFIA) created the Riding Mountain Eradication Area (RMEA), comprising Game Hunting Areas 23 and 23A which surround RMNP. This split the province into two zones, a bTB accredited advanced zone in the RMEA with the remainder of the province being considered bTB-free.

The Task Force subsequently added a Scientific Review Committee (SRC) in 2004 which was chaired by an independent biologist to review science aspects of bTB implementation and a Stakeholder Advisory Committee (TBSAC) in 2003 to provide meaningful input by key stakeholders into implementation plans. The vision of the TB Task Force is to eradicate bovine TB from the GRME with long-term goals of; 1) achieving and maintaining bovine bTB-free

status in domestic cattle, 2) eradicating bovine TB in wildlife that pose a risk to agriculture, and 3) minimizing wildlife-livestock interactions in the Riding Mountain region and unnatural cervid herding behavior which occurs where cervids feed on agricultural produce, thereby minimizing the potential for disease transmission (Tuberculosis, 2002). A part-time Bovine TB Coordinator position was created in 2012 to coordinate interagency cooperation in completing implantation plans and a Policy Steering Group was created from the four government agencies to allow vertical integration of decision making.

Some of the major aspects of implementation plans since 2000 have included; 1) barrier fencing of stored hay on farms within 10 kilometers of the RMNP boundary, 2) increased hunting opportunities in the RMEA by extending hunting seasons for elk into January and February and issuance of additional white-tailed deer permits in portions of the RM of Grandview and Rossburn, 3) elk and white-tailed deer population reductions through a combination of hunting and culling, 4) targeted culling of test positive elk and deer within RMNP, 5) a ban on baiting and feeding of wildlife for purposes of hunting within the RMEA, 6) prescribed burning within RMNP to improve elk habitat and reduce immigration outside of the park onto agricultural lands, and 7) on-farm risk assessment on individual farms within the RMEA to allow use of targeted mitigations such as livestock protection dogs and other risk reduction measures.

Since 2000, active on-farm surveillance has been conducted in the RMEA using caudal fold test screening on live cattle older than 6 months and confirmatory testing using either a gamma-interferon test (Bovigam®) or a comparative cervical test. Between 2000 and 2011, approximately 220,400 cattle, bison and domestic cervids in over 2,600 herds were tested in the RMEA with 6 positive cattle herds being found, the last one in 2008. Risk-based testing of cattle

herds has been conducted annually since then and a scenario tree model for determining the probability of freedom from domestic cattle, bison and cervids in the RMEA is in development (Pers. comm. K. Howden CFIA).

1.5 Objectives of Research

The ultimate objective of this thesis is to provide an enhanced understanding of the epidemiology of *M. bovis* in the GRME and provide a scientific basis for managing the disease from a systems perspective both now and into the future. It is almost certain that other wildlife reservoirs of *M. bovis* will emerge in the future with management being complicated and difficult. A thorough understanding of the dynamics of persistence and ultimate eradication of *M. bovis* from the GRME will provide a unique case example and learning opportunity for managing wildlife reservoirs of disease in wildlife populations.

This study primarily utilizes data collected through targeted wildlife surveillance collected between 1997 and 2013 in order to synthesize and understand the epidemiology of *M. bovis* in wildlife in the GRME. Data on *M. bovis* infection in cattle is also utilized to understand intra-specific transmission at the interface between wildlife and domestic animals. The primary analytic techniques used in this thesis derive from the epidemiological perspective and most of these data are observational in nature, rather than prospectively designed studies. As a result, objectives of the research are presented by chapter, rather than a priori hypotheses, which are traditionally presented in scientific doctoral theses.

The objective of Chapter 2 is to describe the lesion distribution and individual characteristics of *M. bovis* infected wildlife from the GRME, using descriptive epidemiology and contingency table analysis. This will provide the underpinning to compare epidemiological characteristics of the GRME outbreak to other wildlife reservoirs of *M. bovis* both in North America and

worldwide, as each outbreak seems to have both common factors as well as unique characteristics which are described within an overall episystem (O'Connor et al., 2012).

In chapter 3, three blood-based tests that have been used on an experimental basis in the GRME to diagnose *M. bovis* in live elk and white-tailed deer are compared to understand their performance characteristics in these species, as some of these tests have not been described or analyzed previously. Diagnosis of *M. bovis* is particularly challenging in wildlife species and understanding their performance characteristics will help maximize their performance for use in the field and ultimately refine these tools so that they can be used to eradicate *M. bovis* from this episystem. This work also provides key information for providing refined estimates for risk factor analysis in Chapter 4 and an understanding of when *M. bovis* will likely be eradicated from the GRME in the future using scenario tree modeling in Chapter 6.

Chapter 4 will examine the set of risk factors that predict the characteristics of individual elk and white-tailed deer being culture positive for *M. bovis* using logistic regression. This will provide an understanding of the key factors which have led to the control and will ultimately lead to the eradication of *M. bovis* in the GRME in the near future.

Chapter 5 provides an exploratory spatial analysis of the distribution of *M. bovis* in cattle, elk and white-tailed deer in the GRME as well as the characterization of the molecular epidemiology of *M. bovis* isolates from these three host species.

Chapter 6 examines potential future surveillance scenarios in wildlife populations to determine when *M. bovis* could be considered eradicated from the RMEA and current probabilities of disease freedom through scenario tree modeling. It is likely that eradication from this wildlife reservoir could be considered in the very near future (5 to 10 year timeframe) as two years of negative surveillance, despite intensive surveillance of elk and white-tailed deer

populations occurred in 2011/2012 and 2012/2013. This would be one of the first areas in the world to achieve eradication in an established wildlife reservoir of *M. bovis* without eradication of the main reservoir species.

Chapter 7 provides an overall synthesis of the thesis providing a synopsis of the current epidemiological knowledge in the GRME as well as lessons learned through cooperative management of this protracted outbreak to enable other agencies to better prevent and control *M. bovis* outbreaks in wildlife in the future.

1.6 References

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Table 1.1 Major worldwide wildlife reservoirs of *Mycobacterium bovis*.

Region/Country	Sub-region	Major wildlife reservoir host(s)	Spillover host(s)	Epidemiological features/ Major Risk factors
Canada	Wood Buffalo NP	Wood Bison	Moose, black bear	Isolated wild bison population, endemic, bovid reservoir host, co-infected with *Brucella abortus.*
	Riding Mountain NP	Elk (wapiti)	White-tailed deer, wolves, cattle	High elk density, baiting and feeding in 1990's, close contact with infected cattle herds over winter hay bales
USA	Michigan	White-tailed deer	Cattle, elk, raccoons, coyotes, foxes, humans, birds	High WT deer density, baiting for hunting widely prevalent, high percentage of private land in core zone
	Minnesota	White-tailed deer	Cattle	Low WT deer density, spillover from infected cattle herds circa 2007, limited spillover and rapid control in WT deer due to sharpshooting and buyout of cattle farms in core zone
British Isles	Great Britain/Wales	European badger	Cattle, dogs, cats, llama, humans, seals	Social perturbation due to proactive badger culling, increasing incidence, high proportion latency infected badgers, high badger density
	Republic of Ireland(ROI)/ Northern Ireland	European badger	cattle	Reactive badger culling more effective than UK, decreasing incidence, low badger density,
Europe	Spain/Portugal	Red deer, wild boar, cattle		High densities wild boar/deer, wildlife managed for hunting, congregation at waterholes, multiple wildlife reservoir hosts, endangered species (Iberian Lynx) complicates management

	France	Red Deer, Wild Boar	Cattle	High density populations of red deer/wild boar, country remains OTF
	Italy, Austria	Red deer	Cattle	*M. caprae* isolates, high densities of Red Deer are likely reservoir
Africa	South Africa	African buffalo, greater kudu, meerkat	Lion, cheetah, leopard, baboons, , hyena, genet, warthog, bushpig, eland	Expanding range in wild reservoir species, population impacts on carnivores, multiple reservoir species, other diseases (FMD, brucellosis)
	Zambia	Kafue Lechwe	Cattle, humans	Kafue Lechwe considered endangered, high prevalence (~30%), *M. tuberculosis* also found in cattle and humans
New Zealand		Brush-tailed possum (BTP), ferret	Red deer, wild boar, ferret, cattle	Control through population reduction of BTP, Officially bTB Free in 2013, ferrets & pigs used as sentinel species (spillover), Red deer considered spillover species

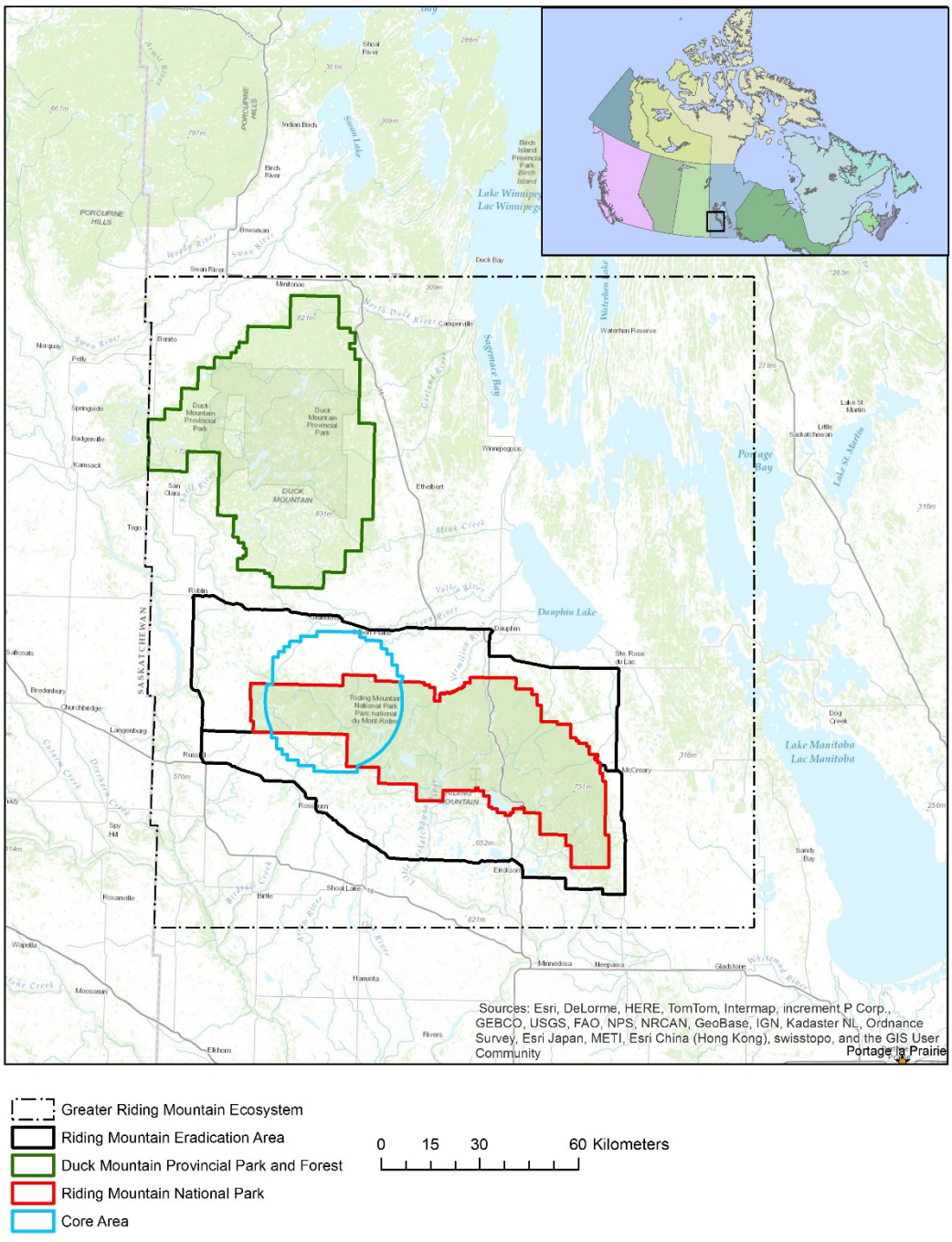

Figure 1.1 Overview map of study area (Greater Riding Mountain Ecosystem) and major jurisdictional zones

CHAPTER 2: LESION DISTRIBUTION AND EPIDEMIOLOGY OF *MYCOBACTERIUM BOVIS* IN ELK (*CERVUS CANADENSIS*) AND WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) IN SOUTHWESTERN MANITOBA, CANADA

The research described in Chapter 2 provides a preliminary analysis of necropsy findings from wildlife surveillance conducted in the GRME between 1997 and 2010. The data provides a comprehensive overview of the epidemiological situation in wildlife in the GRME in terms of prevalence, spatial distribution of cases, preliminary analysis of risk factors in wildlife and lesion distribution and mycobacterial species richness in these species. Chapter 5 extends the information presented here by examining molecular and spatial aspects in more detail using more sophisticated methods, while Chapter 4 builds on this data using advanced regression techniques to further explore the major risk factors associated with bTB culture positive wildlife.

Shury TK and D. Bergeson. 2011. *Lesion distribution and epidemiology of bovine tuberculosis in elk and white-tailed deer in southwestern Manitoba, Canada*. Vet. Med. Int., doi: 10.4061/2011/591980. Epub 2011 Jun 5. PubMed PMID:21776351; PubMed Central PMCID: PMC3135165

Shury conducted all analyses, collected much of the field data, designed the study and wrote the draft manuscript. Bergeson assisted with study design, data collection and manuscript review.

2.1 Introduction

Riding Mountain National Park (RMNP) is a 2,974 square kilometre protected area that is part of a large elevated escarpment that is part of a UNESCO (United Nations Educational, Scientific and Cultural Organization) Biosphere Reserve. This area, which includes the Duck Mountain Provincial Park and Forest (DMPPF) is an important core habitat for a large population of elk (*Cervus elaphus)*, moose (*Alces alces*), white-tailed deer (*Odocoileus virginianus*), wolves (*Canis lupus*) and black bears (*Ursus americanus*) that is considered a southern extension of the boreal forest in Canada. Both protected areas are essentially surrounded on all sides by agricultural landscapes which include forage crop production, grain farming and livestock production. Cattle were grazed sympatrically with wildlife within RMNP and the DMPPF until 1970 when cattle grazing was discontinued in both areas (Wobeser, 2009). Fourteen cattle herds

have been found to be infected with bovine tuberculosis (bTB) since 1991 in the area around RMNP and several of these have been closely linked to cases of infected deer and elk (Lees et al., 2003; Koller-Jones et al., 2006). Local cattle producers have been involved with intensive live cattle testing and movement restrictions, resulting in negative economic consequences for these producers. The two Manitoba Game Hunting Areas that surround RMNP were designated a special management zone called the Riding Mountain Eradication Area (RMEA) in 2003. Following extensive live cattle testing for three years, cattle herds within this zone were subsequently deemed to be bTB-free according to Canadian livestock standards in August of 2006. One additional herd breakdown in cattle was found within the RMEA in May of 2008 (Wobeser, 2009), but no infected cattle herds have been identified after extensive follow-up testing since that time. All *M. bovis* isolates to date from cattle, deer and elk share two closely related spoligotypes designated MB-1 and MB-2 including two infected wolves found within RMNP in 1978 (Lutze-Wallace et al., 2005a; Lutze-Wallace et al., 2005b). It is likely that wildlife species were initially infected as a result of contact with infected cattle, but the infection has likely spilled back to cattle since that time.

Emerging wildlife reservoirs of *M. bovis* infection have created serious negative socioeconomic consequences in the past 15 years in Europe, North America and New Zealand, particularly when the wildlife reservoir has significant conservation or societal value (de Lisle et al., 2002). Determination of disease burden and of species acting as reservoirs is particularly challenging with infected wildlife populations. Reservoir hosts for *M. bovis* are those species that can maintain infection independently through intraspecific transmission without reinfection from another species, while spillover hosts require re-infection from another species to maintain the infection and typically do not maintain the infection in wild populations (de Lisle et al., 2002;

Gavier-Widen et al., 2009). Some species may act as either reservoir or spillover hosts or both, depending on demographic and population specific factors such as population density, presence of artificial feeding, and host immunity (Haydon et al., 2002; Hickling, 2002; O'Brien et al., 2008) and species may form reservoirs in combination (Haydon et al., 2002). In North America, white-tailed deer have been demonstrated to be a competent reservoir species in Michigan, USA while elk are considered a spill-over host (O'Brien et al., 2006; O'Brien et al., 2008). A separate, unrelated outbreak of *M. bovis* is currently occurring in white-tailed deer in the state of Minnesota, but the disease does not appear to be spreading rapidly and deer-to-deer transmission may not be occurring in this state (Hartmann, 2010). The epidemiology of bTB has been described for wild red deer in New Zealand (Lugton et al., 1998; Nugent, 2005) and Spain (Vicente et al., 2007a; Vicente et al., 2007b; Gortazar et al., 2008), but very few references describe the epidemiology or prevalence in wild elk from North America (Lees et al., 2003; O'Brien et al., 2008). The enzootic described in this paper is even more challenging from a disease control perspective as the wildlife that make up the likely reservoir species are found within two environmentally sensitive protected areas (RMNP and DMPPF). Hunting or direct culling have typically been used as a management tools to control wildlife host density and provide samples for disease surveillance, but hunting is not currently permitted within RMNP, making disease management at a landscape scale extremely challenging (Nishi et al., 2006; Gortazar et al., 2008; Wobeser, 2009). This area is one of the last known reservoirs of *M. bovis* in Canada (Nishi et al., 2006), and little is known about the status of this infection in elk and deer in this area.

This study reports on preliminary pathologic findings, lesion distribution and descriptive epidemiology from the area around RMNP and DMPPF for both white-tailed deer and elk and

provides a brief analysis of *M. bovis* confirmed cases found since 1997 in this area. Prevalence and distribution data will be presented allowing a comprehensive assessment of this long-term wildlife reservoir and a discussion of implications for future management and eradication of the disease in wildlife.

2.2 Methods & Materials

2.2.1 Sample collection

Mycobacterium bovis infection was initially discovered in wild ungulates from the RMNP area in a hunter-killed bull elk in 1992, but formal surveys were not initiated until 1997 when hunter harvested elk were collected on the borders of RMNP (Lees et al., 2003; Wobeser, 2009). Data for this study includes deer and elk collected in the RMNP and DMPPF areas through four primary sources; 1) hunter killed elk and deer collected as part of *M. bovis* surveillance efforts between November 1997 to January 2010 (hunter sample), 2) elk and deer collected as part of a blood testing program within RMNP from February 2002 to May 2010 (blood test sample), 3) ground-based culls which were conducted to reduce elk and deer density and determine *M. bovis* prevalence in March 2004 (white-tailed deer only) and a February/March 2009 cull involving both elk and deer (cull sample), and 4) targeted surveillance samples which were collected opportunistically (roadkills, predation, winter kills) and those animals destroyed because they were exhibiting clinical signs of illness (opportunistic sample). Hunter submissions typically consisted of both head and lung samples from harvested animals, but samples occasionally consisted of only the head or lungs. Blood testing was carried out through live animal capture and testing to detect antibodies and cell-mediated immunity to *M. bovis* (detail provided below).

A cull involving local landowners and Manitoba Conservation staff involving white-tailed deer was carried out in March of 2004 through ground-based shooting of deer in areas bordering RMNP. In 2009, culls for population reduction and surveillance were carried out within RMNP

and involved helicopter net gun capture followed by euthanasia with captive bolt gun. All culled animals were transported intact to a laboratory where a full necropsy was conducted on each carcass. Head and lung samples from hunter killed animals were examined at the same laboratory (detail provided below). Targeted surveillance samples were collected opportunistically as a result of public reports and follow-up of predator kills for other research projects. White-tailed deer and elk were considered *M. bovis* positive if they were determined to have a positive culture on any tissue cultured for post-mortem analysis.

Elk and deer captured for blood testing were primarily captured within two protected areas in south western Manitoba, Canada: RMNP and the DMPPF. Animal capture was carried out using helicopter net gunning between February 2002 and May of 2010 during winter and early spring (December to early June, Figure 2.1). Elk were selected haphazardly by the helicopter crew in selected regions within RMNP and DMPPF, but virtually all elk and deer capture for blood testing occurred within these two protected areas. All captured elk were blindfolded and hobbled for short duration (10-15 minutes) and were released immediately after sampling and application of a VHF or GPS collar to allow subsequent relocation and recapture. A cotton spacer made of fire hose was attached to the collar belting to cause them to fall off within 3-6 months after capture. Sixty millilitres of whole blood was collected by jugular venipuncture and placed in either 10 ml sterile glass vials containing no additive, lithium heparin (Vacutainer®) or silicone coating (Vacutainer SST®). Samples without anticoagulant were allowed to clot at room temperature and centrifuged at 3,000 rpm for 15 minutes. For the period 2004 to 2010, three blood based assays were used to detect potentially infected cervids; a lymphocyte stimulation test (LST), a fluorescence polarization assay (FPA) (Surujballi et al., 2009) and a chromatographic immunoassay (Cervid Stat-Pak™)(Lyashchenko et al., 2008). An experimental

polymerase chain reaction (PCR) test was also utilized on buffy coat samples in 2002 to 2004 in addition to these three tests, but it was discontinued in 2005. Serum for the Cervid Stat-Pak evaluation was harvested and frozen at –20° C or tested immediately in some cases. Fresh whole blood with and without anticoagulant were stored at room temperature and shipped immediately upon collection to the Canadian Food Inspection Agency, Mycobacterial Diseases Centre of Expertise (MDCE), Ottawa, Ontario for evaluation using the LST and FPA respectively. Elk testing positive (parallel interpretation) on any one of these diagnostic tests (FPA, LST, Stat-Pak) were subsequently recaptured up to two months later using the methodology described above, euthanized with a captive bolt gun and slung by helicopter to a central laboratory for immediate necropsy. Elk testing negative to three of the four tests (LST, FPA and RT) were not recaptured, but were monitored by aerial telemetry until their radio collars fell off within 3-12 months after capture. A subset of animals that were culled and were tested retrospectively were used to validate the sensitivity of the parallel testing protocol, so very few of these animals were likely truly bTB positive (Shury et al., 2014). Parallel testing involving multiple tests increases the sensitivity while sacrificing specificity, resulting in numerous false positives, but few false negatives (Thrusfield, 2005).

Hunter sampled elk and white-tailed deer heads and lungs were collected annually between September 1997 and January 2010 from voluntary submissions by local hunters through regular and extended hunting seasons. Submissions have been mandatory since 1999 in the RMEA and since 2000 in the Duck Mountain Provincial Park and Forest. All submitted heads and lungs were examined grossly, with specific lymphoid tissues being sent for mycobacterial culture and histopathology prior to the fall/winter of 2001/2002. Since 2002, only tissues from animals exhibiting suspect gross lesions of tuberculosis in lymphoid tissues or palatine tonsils were

submitted for histopathology and mycobacterial culture. Hunting is not allowed within RMNP, but elk and white-tailed deer hunting are allowed within the DMPPF and surrounding area (Figure 2.1). A set of four lymph nodes were routinely evaluated from the head (medial retropharyngeal, parotid, submandibular and lateral retropharyngeal) as well as the palatine tonsils. Lymphoid tissues were sliced thinly at 3-5 mm thickness to look for lesions typical of *M. bovis* and formalin-fixed tissue and fresh tissues were sent to the Mycobacterial Diseases Centre of Excellence (MDCE) laboratory in Ottawa, Ontario. Lung tissues were examined similarly with trachea-bronchial and mediastinal lymph nodes being specifically targeted while lungs were palpated for abnormalities and sliced at 5 cm intervals to check for grossly visible lesions.

Elk and deer sampled opportunistically included predator killed animals, road killed animals, poaching investigations, winter killed animals, or animals observed with unusual clinical signs that were euthanized for necropsy. These animals were either necropsied in a laboratory or in the field depending on location.

2.2.2 Post-mortem and Laboratory procedures

For animals that tested positive on one or more blood tests and for the culled elk and deer, multiple tissues were collected at necropsy as part of a detailed post-mortem collection procedure similar to that collected for other studies involving European badgers(*Meles meles*) (Chambers et al., 2008) and subjected to mycobacterial culture, acid fast staining and histopathological examination. Peripheral lymphoid tissues examined and collected were submandibular, medial and lateral retropharyngeal, parotid, palatine tonsil (tonsillar crypt), prescapular, popliteal, prefemoral, supramammary/testicular, internal iliac, hepatic, portal, mesenteric, bronchial, and mediastinal lymph nodes. Pools of tissue from body, head, abdominal and thoracic lymph nodes were submitted for mycobacterial culture regardless of whether gross lesions were seen at necropsy or not. All other organ systems were systematically examined for

gross lesions indicative of mycobacteriosis and any suspect tissue was also sent for mycobacterial culture, histopathological evaluation, and PCR testing to confirm identity of cultured mycobacteria. Harvested tissues were either frozen at –20° C or refrigerated and were shipped to the MDCE within 24 to 48 hours of collection. Formalin-fixed tissues were embedded in paraffin, cut into sections 5 mm thick, and stained with hematoxylin and eosin as well as by the Ziehl–Neelsen technique for detection of acid-fast bacilli. Slides of the tissue sections were examined by a pathologist experienced in the diagnosis of bTB. The tissues were cultured on multiple solid culture media for mycobacteria using the method described by Rohonczy et al (Rohonczy et al., 1996). Inoculated media were incubated at 37° C for 12 weeks and examined every 2 weeks for evidence of bacterial growth.

Elk and deer were considered bTB positive if they had a positive culture for *M. bovis* on any tissue submitted for culture (Surujballi et al., 2009). Spoligotyping to type cultured bTB complex organisms was conducted as described previously (Lutze-Wallace et al., 2005b). Ages of hunter killed elk and deer at necropsy were determined by estimation of tooth wear into one of five age categories; less than one year of age, one to two years of age, three to five years of age, six to eight years of age, or greater than 8 years. Elk and deer that were culled, blood tested or found opportunistically were aged by examination of tooth sections and counting cementum annuli (Keiss, 1969).

2.2.3 Statistical analysis

Sampled elk and deer were grouped based on sampling location into one of four risk zones created to monitor the prevalence and distribution of *M. bovis* in wildlife (Figure 2.1). Prevalence was estimated using the methods described in Thrusfield (2005) and 95% confidence intervals were estimated using WINPEPI software version 10.1 using Wilson's score method (Wilson, 1927). Trend analysis on prevalence data was conducted using WINPEPI software

using a two-way Cochrane-Armitage test for trend with Fishers exact 95% confidence intervals. Analysis of the proportion of culture positive animals with gross visible lesions in different tissues were compared using Upton's modified (N-1) Chi-square (Campbell, 2007).

2.3 Results

The overall prevalence of *M. bovis* infection in elk and white-tailed deer has been consistently very low in the area in and around RMNP during the period of this survey (Figure 2.2). Mean *M. bovis* culture positive period prevalence over the twelve year surveillance period was 0.89% (0.66% – 1.21%) for elk and 0.15% (0.08% – 0.27%) for white-tailed deer. A total of 41 culture positive elk out of 4,583 and 11 culture positive white-tailed deer out of 7,379 were detected through all forms of surveillance. Elk prevalence has varied quite dramatically from year to year with the highest prevalence being detected in the winter of 2002/2003 (2.01%, Fig. 2) when 10 culture positive animals were found through blood testing within RMNP. Prevalence in white-tailed deer has been consistently below 1% throughout this period.

Virtually all infected elk and white-tailed deer have come from a small geographic area around the north-western border of RMNP (Table 2.1, Figure 2.1). This 1800 km^2 area designated the Western Control Zone, where most management activities have been focussed, encompasses 37 of the 41 (90.2%) culture positive elk and 10 of 11 (90.9%) culture positive white-tailed deer found through all forms of surveillance since 1997. Prevalence of *M. bovis* within the Western Control Zone has been consistently higher than other surveillance areas ranging from zero to 6.85% (Table 2.1). Elk from the WCZ were approximately 21.1 times more likely (χ^2= 67.7, $p < 0.001$) to be culture positive than elk from outside this area and white-tailed deer were approximately 49.1 times more likely (χ^2= 56.4, $p < 0.001$) to be culture positive compared to deer from outside this zone (based on pooled data from the other three zones for comparison).

There was no evidence of a temporal linear trend in overall prevalence for elk (p=0.827), deer (p=0.80) or both species combined (p=0.363) when all data from 1997 to 2010 was examined. But if only the data from 2003 to 2010 was examined neither elk (p=0.120) nor deer (p=0.768) exhibit a linear trend, but both species combined exhibit a significant downward trend (p=0.019) in this most recent time period, as can be observed in Figure 2.2. This time period also corresponds to a significant decline in number of elk and deer examined, although prevalence and sample numbers were not correlated (ρ=-0.093).

Mycobacterium bovis was the most common mycobacterial isolate cultured from elk, but *M. terrae* was the most frequent isolate from white-tailed deer (Table 2.2). *M. avium* was only cultured from elk, while *M. kansasi* was only cultured from deer. Other mycobacteria isolated included *M. fortuitum* and *M. chelonae*. All mycobacteria including *M. bovis* were most frequently isolated when the entire carcass was available for examination compared to other tissues such as the head or lungs. Thirty-one culture positive elk and 5 culture positive deer were diagnosed from examination and full necropsy of the entire carcass. Of these, 19 of 31 (61.3%) elk had gross visible lesions in the head, 15 of 31 (48.4%) had gross visible lesions in the lungs, and 25 of 31 (80.6%) had gross visible lesions in either the head lymph nodes or lungs. Three of 5 (60%) culture positive deer which had full necropsies had gross visible lesions in the head, 0 of 5 had gross visible lesions in the lungs, and 3 of 5 (60%) had gross visible lesions in either the head lymph nodes or lungs.

The most common sites of gross lesions in culture positive elk were the lungs, palatine tonsils and retropharyngeal lymph nodes, while in white-tailed deer it was the retropharyngeal lymph node, abdomen (mesenteric lymph node) and body lymph nodes (popliteal)(Table 2.3). All (100%) culture positive white-tailed deer and elk exhibited at least one gross lesion compatible

with *M. bovis* infection at necropsy. Gross lesions typically consisted of caseo-purulent or granulomatous lesions which were either multi-focal or singular and were commonly associated with some degree of mineralization. Histologically, lesions were typically well encapsulated when in lymphoid tissues and were often disseminated when in the lungs. Male elk were approximately four times more likely to have gross visible lesions in the lungs compared to female elk when stratified by sex (Table 2.4). Gross lesions did not vary significantly by sex for other tissues examined.

Neither sex was more likely to be *M. bovis* culture positive for both elk and deer based on proportions sampled in this study (Table 2.5). The prevalence of infection (*M. bovis* culture positive) increased with age class in elk, but the oldest age class of deer (>8 years) had very few samples (n=25) and no *M. bovis* positive animals. The majority of culture positive elk and deer were detected through blood testing, followed by opportunistic sampling and culling (Table 2.5), while fewer culture positive animals were detected among hunter-killed animals. At necropsy, blood-tested elk had odds of testing culture positive of 10.6 compared to hunter-killed elk, while blood-tested deer had odds of 13.6 compared to hunter-killed deer (Table 2.5). The seven culture positive elk in the younger age classes (less than or equal to 2 years of age) were all found prior to 2004, and no elk younger than five years of age has been found since then.

2.4 Discussion

Bovine tuberculosis has been consistently present in elk in the RMNP ecosystem since at least 1992 and in white-tailed deer sporadically since 2001. Culture positive elk have been found every year in this area with the exception of two years (1997/1998 and 2001/2002), while infected white-tailed deer have been detected only in certain years and not consistently from year to year despite testing large numbers of animals (6 to 1474 deer annually). For this reason, it has been suggested that elk are the primary reservoir species of *M. bovis* within this ecosystem (Lees

et al., 2003). The factors which result in this differential temporal occurrence could be related to differences in social structure, susceptibility to *M. bovis*, individual contact rate, herd immunity and method of testing.

Studies in Michigan and New Zealand suggest that elk or red deer do not act as reservoir species, but are spillover hosts instead (Nugent, 2005; Zanella et al., 2008b; O'Brien et al., 2009) while data from red deer in France suggest they may act as a reservoir host in association with wild boar (Zanella et al., 2008a; Zanella et al., 2008b). Data presented in this study suggest that elk may be a primary reservoir species, but that infected white-tailed deer may also be necessary to maintain ongoing infection in a multi-species reservoir system (Haydon et al., 2002). Infected cattle herds may also be a necessary part of this multi-species reservoir, as infected cattle herds have not been found consistently in this area despite rigorous and intensive testing (Koller-Jones et al., 2006; Wobeser, 2009), but the role of cattle as a reservoir species is currently undetermined. It is unlikely that there are other undetected reservoir species in this ecosystem as multiple species have been assessed with negative findings to date (Koller-Jones et al., 2006; Sangster et al., 2007; Wobeser, 2009).

This study provides some evidence that overall prevalence of *M. bovis* in both deer and elk is declining since 2003 as the number of infected cattle herds has also declined. Another piece of evidence which supports this is the lack of younger age classes of elk found positive since 2004. Since 2004, all *M. bovis* positive elk found through surveillance activities have been 5 years of age or older, but prior to 2004, five elk that were 2 years of age, 1 yearling and one calf were found to be infected. This trend is not apparent for white-tailed deer as the two most recent infected white-tailed deer were 2 years of age.

Since *M. bovis* infection in cervids results in chronic disease and elk are a relatively long-lived ungulate species, especially in a protected area, it is likely that positive cases of *M. bovis* will continue to be detected in both elk and deer in this area for several years to come. The net force of infection is the instantaneous per capita rate that individual cervids become infected (Caley et al., 2009). This can be estimated in wild populations infected with *M. bovis* using the proportion of young age classes found infected on cross-sectional surveys (Nugent, 2005), as these represent relatively new infections based on short exposure times. Based on the findings of this study, the net force of infection has decreased in elk since 2004. Similar to previous studies of both red deer and white-tailed deer, age specific prevalence of *M. bovis* increases dramatically in older age classes of both elk and deer (Lugton et al., 1998; O'Brien et al., 2002; Vicente et al., 2007b). Elk older than 6 years were 10 times more likely to be culture positive compared to younger age classes. Small numbers of positive deer made this association much less apparent with white-tailed deer, but the trend was similar.

The prevalence of *M. bovis* in wild elk is significantly lower in this ecosystem than in comparable populations of red deer found in other parts of the world including New Zealand, Spain and France, where prevalence often exceeds 30%. Spatial aggregation at waterholes has been shown to be an important risk factor for infection in Spanish red deer (Vicente et al., 2007b), while association with other infected wildlife reservoirs such as brush-tailed possum and wild boar have been shown to be important risk factors in New Zealand and France, respectively (Ryan et al., 2006b; Zanella et al., 2008a; Zanella et al., 2008b). The role of host density in maintenance of cervid reservoirs of *M. bovis* is somewhat equivocal with some studies finding density dependent effects, while others have refuted this hypothesis (Vicente et al., 2007a; Conner et al., 2008; Wobeser, 2009).

Attempts to model *M. bovis* infection in wild ungulates have relied upon on density-dependent transmission (McCarty and Miller, 1998) and some studies have found positive correlations between density and prevalence (Vicente et al., 2007a). Supplemental feeding and spatial aggregation around waterholes have been positively associated with spatial occurrence of *M. bovis* (Hickling, 2002; Vicente et al., 2007b), suggesting that contact structure and localized congregations may be important factors allowing maintenance and transmission of the disease in wildlife reservoirs. Elk densities were historically much higher in the RMNP area (Zhao, 2006) and deer densities have likely been increasing since the early part of the twentieth century, when white-tailed deer began colonizing this area.

One of the management strategies instituted in 2003 to control *M. bovis* in this area was an attempt to keep the regional elk population at historically low levels in an attempt to reduce transmission (Nishi et al., 2006). Other strategies introduced at roughly the same time were lengthened hunting seasons, a moratorium on regional wolf trapping, and fencing of hay storage yards around RMNP (Wobeser, 2009; Brook, 2010). It appears that this combination of management factors has likely played a role in reducing the prevalence of *M. bovis* in ungulates in the RMNP area since 2003, as well as a decreasing the number of spillover events to surrounding cattle herds. Strategies to eventually eliminate bovine tuberculosis in this ecosystem are being actively considered by government agencies and local stakeholders.

The pathology of *M. bovis* infection found in elk is similar to that described in both captive and farmed elk as well as wild red deer populations in other parts of the world, with the exception that all culture positive elk had grossly visible lesions, meaning there were no culture positive elk without visible lesions (NVL) in this study. Other studies of wild red deer in Spain and New Zealand have found proportions of culture positive elk that are NVL as high as 30%

(Gavier-Widen et al., 2009),(Lugton et al., 1998), while studies in Canadian captive elk had proportions of approximately 7% (Rohonczy et al., 1996). The reason for this difference may be that a significant proportion of elk in this study were examined using a detailed necropsy procedure that was designed to find *M. bovis* lesions, whereas other studies have typically used field necropsies or only examined portions of carcasses. Thus, many subtle lesions that may have been missed on a field necropsy were discovered during this study.

Other mycobacteria isolated from lesions in both elk and deer likely decrease the specificity of diagnostic tests for mycobacteria. *M. terrae* was the most common mycobacterial isolate in white-tailed deer, but previous studies have not reported isolation of *M. terrae* commonly (Fitzgerald et al., 2000). *M. avium* was the next most common mycobacterial isolate in elk. Prior exposure to environmental mycobacteria such as *M. terrae* and other mycobacteria may play a role in sensitizing the host immune response to *M. bovis* (Buddle et al., 2002; Nol et al., 2009) and may be one factor causing individual heterogeneity in rates of infection and resistance in wild populations.

Both male elk and white-tailed deer were more likely to be culture positive for *M. bovis*, but the difference was not significant due to low sample sizes when stratified by species (Table 2.5). Males have generally had higher odds of testing positive to *M. bovis* in studies of both red deer and white-tailed deer (O'Brien et al., 2002; Lees et al., 2003). In the RMNP ecosystem, 10 of 11 culture-positive white-tailed deer have been male since 2001, but the low numbers of positives and higher proportion of male deer in the sample dilutes this effect.

Sampling zone and surveillance method were significantly associated with *M. bovis* status in this study with animals being sampled in the Western Control zone being at a significantly higher risk of being positive for *M. bovis* than elk or deer sampled in other areas. Both elk and

deer sampled through blood testing and culling were much more likely to be culture positive than animals sampled through hunting or other surveillance methods. One reason for this is that once *M. bovis* positive elk were found in the Western Control zone through blood sampling, surveillance efforts tended to focus on this area to a certain degree, increasing the likelihood of finding culture positive animals. Hunter samples tended to be more randomly distributed but are limited spatially in that none came from within RMNP.

The true extent of *M. bovis* infection in this ecosystem was not fully realized until a costly and rigorous sampling program was carried out using blood tests within RMNP. Using multiple surveillance methods rather than relying on a single method was a key determinant in determining the extent of infection in wildlife in this ecosystem. Detection of *M. bovis* in wildlife species at fine spatial scales within protected areas is much more difficult (Gortazar et al., 2008) and this is one of the first studies to rely on blood sampling rather than traditional skin testing and hunter surveillance to determine *M. bovis* distribution in a cervid reservoir.

Similar to Michigan, *M. bovis* appears to be highly clustered in cervids in the RMNP area, but unlike Michigan, elk are more commonly infected than white-tailed deer (O'Brien et al., 2008). Reasons for this discrepancy are unknown, but are likely related to different population densities, social behaviour, and presence of baiting and feeding for hunting (Hickling, 2002) . White-tailed deer densities in Michigan are much higher compared to south-western Manitoba (Wobeser, 2009) and the role of supplemental feeding to bait deer in Michigan (O'Brien et al., 2006) may act to further aggregate deer at local spatial scales. Supplemental feeding and baiting for purposes of hunting have been prohibited through legislation and enforced in the RMEA since 2002. Baiting and feeding is difficult to control in some jurisdictions, but restrictions have been relatively well accepted by local stakeholders in Manitoba. Conversely, elk population size and

density are likely greater within RMNP than is found in Michigan, where elk densities are somewhat lower and not directly within the core area where *M. bovis* is found. Other factors such as habitat quality and quantity, intraspecific and interspecific contact rates, and herd immunity may also play a role in the maintenance of *M. bovis* infection in these wildlife reservoirs. Studies currently ongoing in the RMNP area hope to clarify the role of some of these important factors.

2.5 Conclusions

M. bovis infection has been consistently present in a relatively small geographic area located in and around the north-western part of RMNP since at least 1978, but significant annual variation in prevalence has occurred since 1997 in both elk and deer. Period prevalence in elk is approximately six times higher than deer, suggesting they may be a significant reservoir host of *M. bovis* in this ecosystem, but that infected white-tailed deer may also be required to maintain a true reservoir in this system. Pathological lesions associated with *M. bovis* infection and distribution of those lesions in wild elk and deer are very similar to those described in other parts of the world, but fewer NVL elk were found compared to red deer. The lack of culture positive animals in younger age classes of elk since 2003 indicate that the net force of infection as well as overall prevalence are declining in elk in this area, but further surveillance and monitoring will be necessary to determine if this is consistent over time. This study demonstrates that it is vitally important to sample all geographical sites occupied by *M. bovis* host species using a variety of surveillance methods if possible, or focal aggregations of disease may be overlooked for long periods of time. Both the management and surveillance of infected wildlife reservoirs is challenging and difficult, but blood-based assays were a crucial part of estimating the apparent prevalence and spatial distribution of *M. bovis* infection in this system.

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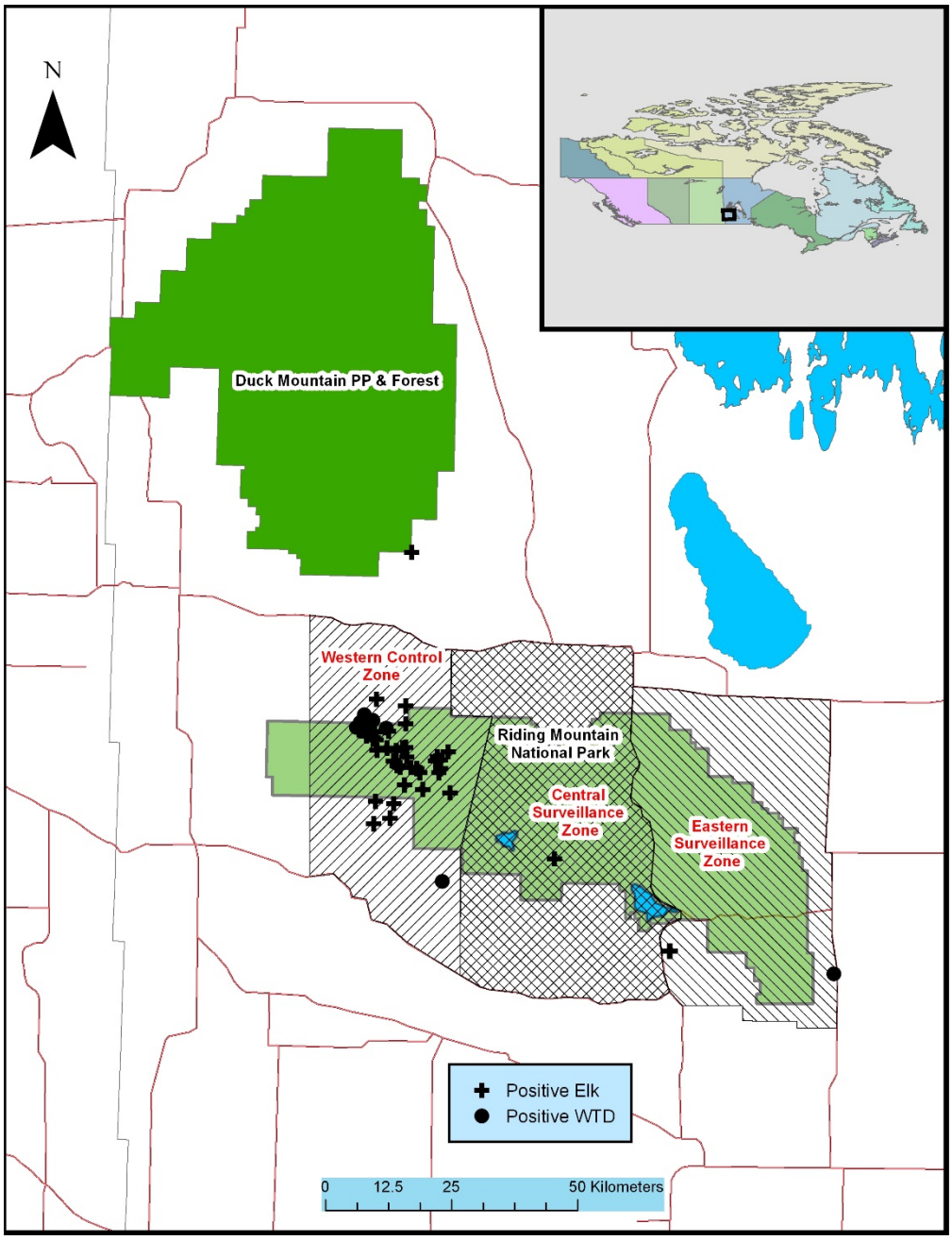

Figure 2.1 Locations of sampling zones and *M. bovis* culture positive elk and white-tailed deer in south-western Manitoba from 1997 to 2010.

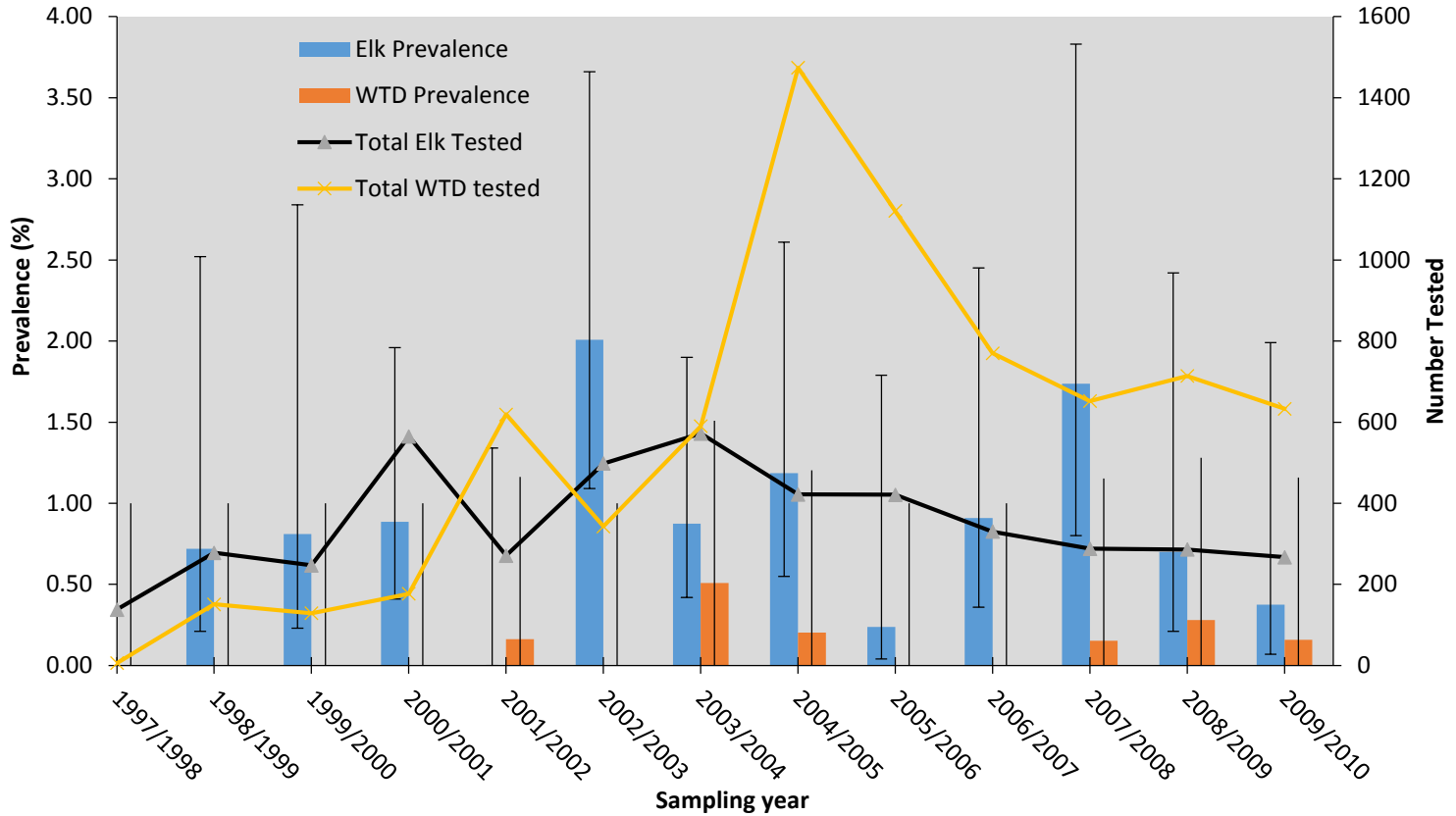

Figure 2.2 Overall estimated annual prevalence (95% confidence intervals on bars) of *M. bovis* and total number of elk and white-tailed deer sampled from south-western Manitoba from 1997 to 2010

Table 2.1 Zone specific prevalence of *M. bovis* in elk and deer from south-western Manitoba from 1997 to 2010.

		Outside RMEA[b]			Eastern Surveillance Zone			Central Surveillance Zone			Western Control Zone		
Species	**Sampling Year[a]**	Prevalence (%)	95% CI	No. Tested	Prevalence (%)	95% CI	No. Tested	Prevalence (%)	95% CI	No. Tested	Prevalence (%)	95% CI	No. Tested
Elk	1997/1998	0	0 - 65.76	2	0	0 - 4.87	75	0	0 - 11.0	31	0	0 - 11.35	30
	1998/1999	0	0 - 13.32	25	0.81	0.14 - 4.43	124	1.72	0.31 - 9.1	58	0	0 - 5.13	71
	1999/2000	0	0 - 6.02	60	1.37	0.24 - 7.36	73	0	0 - 6.02	60	1.85	0.33 - 9.77	54
	2000/2001	0	0 - 3.26	114	0	0 - 2.12	177	0	0 - 3.47	107	2.99	1.29 - 6.82	167
	2001/2002	0	0 - 4.42	83	0	0 - 4.01	92	0	0 - 11.03	31	0.00	0 - 5.58	65
	2002/2003	0	0 - 2.96	126	0	0 - 2.26	166	0	0 - 6.02	60	6.85	3.76 - 12.15	146
	2003/2004	0	0 - 3.89	95	0	0 - 1.83	206	0	0 - 5.35	68	2.46	1.06 - 5.64	203
	2004/2005	0.55	0.1 - 3.05	182	0	0 - 4.28	86	0	0 - 15.5	21	3.01	1.18 - 7.48	133
	2005/2006	0	0 - 1.62	233	0	0 - 5.92	61	0	0 - 5.5	66	1.64	0.29 - 8.72	61
	2006/2007	0	0 - 2.87	130	0	0 - 4.87	75	0	0 - 11.35	30	3.16	1.08 - 8.88	95
	2007/2008	0	0 - 3.43	108	0	0 - 6.42	56	0	0 - 27.75	10	4.39	1.89 - 9.86	114
	2008/2009	0	0 - 4.53	81	0	0 - 25.9	11	0	0 - 27.75	10	1.09	0.3 - 3.88	184
	2009/2010	0	0 - 3.56	104	0	0 - 29.9	9	0	0 - 4.58	80	1.35	0.24 - 7.27	74
WTD[c]	1997/1998	0	- 0	0	0	0 - 56.1	3	0	0 - 56.1	3	0	0 - 0	0
	1998/1999	0	0 - 5.5	66	0	0 - 7.41	48	0	0 - 12.1	28	0	0 - 29.9	9
	1999/2000	0	0 - 10.7	32	0	0 - 7.71	46	0	0 - 8.38	42	0	0 - 29.9	9
	2000/2001	0	0 - 7	51	0	0 - 6.21	58	0	0 - 8.97	39	0	0 - 11.7	29
	2001/2002	0	0 - 0.77	494	0	0 - 6.11	59	0	0 - 11.03	31	2.86	0.51 - 14.53	35
	2002/2003	0	0 - 2.01	187	0	0 - 6.21	58	0	0 - 6.53	55	0	0 - 8.2	43
	2003/2004	0	0 - 3.5	106	0	0 - 1.84	205	0	0 - 3.66	101	1.69	0.57 - 4.84	178
	2004/2005	0	0 - 0.47	828	0	0 - 1.41	268	0	0 - 2.45	153	1.33	0.45 - 3.85	225
	2005/2006	0	0 - 0.61	623	0	0 - 1.79	211	0	0 - 3.05	122	0	0 - 2.28	165
	2006/2007	0	0 - 0.85	448	0	0 - 2.36	159	0	0 - 4.69	78	0	0 - 4.32	85
	2007/2008	0	0 - 0.97	393	0	0 - 3.21	116	0	0 - 13.8	24	0.84	0.15 - 4.61	119
	2008/2009	0	0 - 1	380	0	0 - 2.63	142	0	0 - 8.97	39	1.31	0.36 - 4.64	153
	2009/2010	0	0 - 0.78	488	1.79	0.32 - 9.5	56	0	0 - 12.1	28	0	0 - 5.92	61

a – Sampling year refers to period from July to June split over two calendar years.
b - Outside of Riding Mountain Eradication Area [RMEA].
c – White-tailed deer

Table 2.2 Summary of gross pathological and culture results for infected deer and elk by number of tissues examined from south-western Manitoba.

Species	Tissues Examined	No. Examined (%)	No. Cultured	*M. bovis*		*M. avium*		*M. kansasi*		*M. terrae*		Other Mycobacteria[a]	
				No.	%	No.	%	No.	%	No.	%	No.	%
Elk	Whole carcass	446 (12.3%)	445	31	6.97	5	1.12	0	0.00	4	0.90	2	0.45
	Head & Lungs[b]	2589 (71.5%)	2567	9	0.35	5	0.19	0	0.00	6	0.23	0	0.00
	Head Only	571 (15.8%)	569	1	0.18	1	0.18	0	0.00	0	0.00	0	0.00
	Lungs Only[b]	14 (0.4%)	9	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
	Total	3620	3590	41	1.17	11	0.31	0	0.00	10	0.28	2	0.06
WTD													
	Whole carcass	452 (6.5%)	447	5	1.12	1	0.22	0	0.00	6	1.34	0	0.00
	Head & Lungs[b]	5198 (75.2%)	5176	5	0.10	2	0.04	1	0.02	12	0.23	0	0.00
	Head Only	1208 (17.5%)	1192	1	0.08	1	0.08	0	0.00	0	0.00	0	0.00
	Lungs Only[b]	51 (0.7%)	47	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
	Total	6909	6815	11	0.16	4	0.06	1	0.01	18	0.26	0	0.00

[a] – One isolate was *M. chelonae* and one was *M. fortuitum*

[b]- Lung tissue including tracheobronchial and mediastinal lymphoid tissues.

Table 2.3 Site of gross visible lesions (GVL) in *M. bovis* positive elk and white-tailed deer from south-western Manitoba.

		Medial Retropharyngeal Lymph Node	Parotid Lymph Node	Mandibular Lymph Node	Palatine Tonsil	Lateral Retropharyngeal Lymph Node	Lungs[a]	Body Lymph Nodes[b]	Abdominal Lymph Nodes[c]	No Visible Lesions
Elk	GVL	12	9	2	19	2	20	10	7	0
	Total tested	41	41	41	41	41	40	36	36	41
	Proportion	29.3	22.0	4.9	46.3	4.9	50.0	27.8	19.4	0.0
WTD	GVL	8	1	0	0	0	2	1	2	0
	Total tested	11	11	11	11	11	10	5	5	11
	Proportion	72.7	9.1	0.0	0.0	0.0	20.0	20.0	40.0	0.0

[a]-Includes tracheobronchial and mediastinal lymphoid tissues.
[b] - Includes prescapular, prefemoral, supramammary/testicular, and popliteal lymph nodes.
[c]- Includes mesenteric, hepatic, portal, and internal iliac lymph nodes.

Table 2.4 Proportion of culture positive elk with gross visible lesions (GVL) in different tissues and body sections stratified by sex.

	Lung[a] GVL	Medial Retropharyngeal GVL	Parotid GVL	Tonsil GVL	Abdominal GVL[b]	Body GVL[c]
Male	13/20 (65.0)	6/22 (27.3)	7/22 (31.8)	11/22 (50.0)	4/15 (26.7)	5/15 (33.3)
Female	6/19 (31.6)	6/19 (31.6)	2/19 (10.5)	8/19 (42.1)	4/15 (26.7)	6/15 (40.0)
χ^2	4.24 (0.039)	0.089 (0.765)	2.63 (0.105)	0.249 (0.618)	0.0 (1.0)	0.139 (0.710)
Odd Ratio	4.02 (0.89 – 18.9)	0.81 (0.35 – 2.16)	3.97 (0.60 – 43.5)	1.38 (0.34 – 5.65)	1.0 (0.31 – 3.28)	0.75 (0.33-2.04)

[a]-Includes tracheobronchial and mediastinal lymphoid tissues.
[b]- Includes mesenteric, hepatic, portal, and internal iliac lymph nodes.
[c] - Includes prescapular, prefemoral, supramammary/testicular, and popliteal lymph nodes.

Table 2.5 Prevalence of *M. bovis* in elk and white-tailed deer (WTD) stratified by sex, age category and surveillance method from south-western Manitoba.

			Culture -	Culture +	Prevalence	Odds Ratio	χ^2	p
Elk	Age Category (years)	<1	449	1	0.22	1		
		1 to 2	763	6	0.78	3.53 (0.42 to 162.7)	0.202	0.961
		3 to 5	1721	12	0.69	3.13 (0.46 to 134.1)	1.334	0.248
		6 to 8	494	12	2.37	10.9 (1.6 to 467)	8.19	0.004
		>8	415	11	2.58	11.9 (1.7 to 513.4)	9.01	0.003
	Sex	Female	2342	20	0.85	1		
		Male	1516	22	1.43	1.70 (0.88 to 3.29)	2.98	0.09
	Surveillance Method	Hunted	3345	9	0.27	1		
		Opportunistic	179	4	2.19	8.3 (1.8 to 30.1)	219	<0.001
		Culled	73	2	2.67	10.2 (1.05 to 50.3)	13.2	<0.001
		Blood Test	254	27	9.61	39.5 (17.8 to 96.3)	230.6	<0.001
WTD	Age Category (years)	<1	453	0	0.00	ND	ND	ND
		1 to 2	2001	2	0.10	1		
		3 to 5	4271	3	0.07	0.7 (0.08 to 8.42)	0.151	0.698
		6 to 8	222	6	2.63	27.0 (4.8 to 274.6)	36.7	<0.001
		>8	25	0	0.00	ND	ND	ND
	Sex	Female	1803	1		1		
		Male	5358	10		3.37 (0.48 to 145.8)	1.51	0.22
	Surveillance Method	Hunted	6735	6	0.09	1		
		Opportunistic	195	0	0.00	0 (0 to 29.5)	0.17	0.68
		Culled	224	2	0.88	10.02 (0.98 to 56.4)	12.08	0.001
		Blood Test	71	3	4.05	47.4 (7.5 to 226.3)	87.2	<0.001

[a] – Stratum specific prevalence (Number positive/Total number tested per category).
[b] – Category used as the reference category for odds ratio and chi-square calculations

CHAPTER 3: FIELD EVALUATION AND SEROPREVALENCE OF THREE BLOOD-BASED ASSAYS FOR ELK (*CERVUS CANADENSIS*) AND WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) NATURALLY INFECTED WITH *MYCOBACTERIUM BOVIS*

The research described in Chapter 3 is important to validate the blood testing portion of wildlife surveillance, which provided numerous samples for use in earlier and subsequent chapters. It was important to validate these blood tests, as they had not been used in wild elk or deer populations for diagnosis of M. bovis previously alone or in combination. Blood test negative elk are used for subsequent analyses and so it was important to demonstrate that all three tests when interpreted in parallel had a high negative predictive value in this particular population. These data are subsequently used to describe serological epidemiology of elk and white-tailed deer since 2001 in the GRME.

The elk test validation portion of this manuscript was published in Preventive Veterinary Medicine in August 2014. Copyright is currently held by the Government of Canada. Reprinted from *Preventive Veterinary Medicine* 115 (pp. 109-121), Shury TK, Bergeson D, Surujballi O, Lyashchenko KP, Greenwald R. *Field evaluation of three blood-based assays for elk (Cervus canadensis) naturally infected with Mycobacterium bovis*. Copyright 2014 with permission from Elsevier and the Parks Canada Agency.
Shury conducted all analyses, collected much of the field data, helped design the study and wrote the draft Preventive Veterinary Medicine (PVM) manuscript and independently wrote and analyzed the white-tailed deer validation and seroprevalence portions. Bergeson was involved with study design and implementation and Surujballi performed some of the diagnostic testing and both contributed through editing of the final PVM manuscript. Lyashchenko and Greenwald contributed by editing drafts of the PVM manuscript.

3.1 Introduction

Diagnosis of natural infection caused by *M. bovis* in both wild and domestic animals is challenging due to problems with an imperfect gold standard (mycobacterial culture), long periods of clinical latency, intermittent shedding of the organism and a large range of clinical severity due to disease (de Lisle et al., 2002; Drewe et al., 2009). Diagnostic testing of wildlife reservoirs of *M. bovis* pose an additional challenge due to logistical constraints imposed by requirements for multiple captures, which are required for interpretation of skin-based tuberculin tests commonly used in domestic animals (de la Rua-Domenech et al., 2006) and poor performance of tuberculin based assays in wild species (Waters et al., 2011). Several serologic and blood-based assays have been developed recently which exhibit reasonable diagnostic

performance criteria using a single blood sample (Lyashchenko et al., 2000; Harrington et al., 2008b; Lyashchenko et al., 2008; Drewe et al., 2009; Gowtage-Sequeira et al., 2009; Greenwald et al., 2009; O'Brien et al., 2009; Buddle et al., 2010; Chambers et al., 2010; Himsworth et al., 2010; Schiller et al., 2010). Methods of validating diagnostic tests vary depending on whether animals sampled are alive or dead, and the validity of using mycobacterial culture as a gold standard has been questioned by some authors due to low sensitivity and heavy reliance on the number and quality of tissues examined at necropsy (Greiner and Gardner, 2000; Chambers, 2009; Chambers et al., 2009; Gavier-Widen et al., 2009). Mycobacterial culture has been deemed an imperfect gold standard for validation of new diagnostic tests in wildlife, which tends to underestimate the sensitivity and specificity of newer tests (Drewe et al., 2009). For this reason and inability to get post-mortem samples for culture in many studies, some researchers rely instead on Bayesian analytical techniques which do not require a gold standard but use a latent variable approach (Toft et al., 2007; Clegg et al., 2011). Serological assays have been shown to have many advantages in terms of cost and ease of use in the field, but have also been shown to be not particularly useful or sensitive when used as a sole diagnostic test under field conditions for diagnosis of *M. bovis* (Cousins and Florisson, 2005; O'Brien et al., 2009). The need for large scale studies of naturally infected cervids from low prevalence populations using gold standard validation has been recommended by these authors as well.

The primary objective of this study was to compare test performance in the field of three blood-based assays for the diagnosis of *M. bovis*; a Lymphocyte Stimulation Test (LST), a Fluorescence Polarization Assay (FPA), and an immunochromotographic assay (Cervid TB Stat-Pak™) in naturally infected elk (*Cervus canadensis*) and white-tailed deer (*Odocoileus virginianus*) populations in southern Manitoba, Canada in the vicinity of Riding Mountain

National Park. This wildlife reservoir is maintained primarily by elk with spillover occurring to cattle and white-tailed deer. Infected elk have been present since 1992 and infected white-tailed deer since 2000 and management actions to reduce transmission between infected wildlife and cattle were instituted in 2003, with corresponding reductions in prevalence in elk and deer since that time (Lees et al., 2003; Shury and Bergeson, 2011).

A secondary objective was to compare two widely used diagnostic test validation techniques; 1) the widely accepted standard of comparing the three tests to the gold standard of mycobacterial culture using a two-stage classical approach using post-mortem sampled elk and deer and, 2) more recently applied techniques using a Bayesian latent class analysis which do not rely on a gold standard, using data from live captured elk and deer. These two approaches were contrasted to determine which method was a better validation method for this population. A validation subsample of elk and deer that were blood-tested at the same time as necropsy and culture were also evaluated to determine the degree of validation bias that was present in the classical two-stage sampling strategy. Results of this evaluation were then used to determine the test characteristics of these tests separately and in combination, and to derive the optimum combination of tests for use in ante-mortem diagnosis of *M. bovis* in this and similar populations of wild cervids. Results of these analyses are then used to provide unbiased estimates of prevalence within two subpopulations of elk and white-tailed deer that occur within this wildlife reservoir and to provide wildlife managers with recommendations for sampling from wildlife reservoirs of *M. bovis*.

3.2 Methods

3.2.1 Animal capture and post mortem testing

Elk and white-tailed deer were primarily captured within two protected areas in south western Manitoba, Canada; Riding Mountain National Park (RMNP) and the Duck Mountain

Provincial Park and Forest (DMPPF) (Figure 3.1). Animal capture was carried out using helicopter net gunning between February 2002 and April of 2011 during winter and early spring and is described in more detail in (Shury and Bergeson, 2011). Animal capture and handling was approved under several animal care permits from the University of Manitoba (F01-037), the University of Alberta (472602), and the University of Saskatchewan (#20060067 & #20110031).

Three blood based assays were used to detect potentially *M. bovis* infected cervids including a LST, a FPA, and a chromatographic immunoassay (Cervid TB Stat-Pak™). Serum for the Cervid TB Stat-Pak™ evaluation was harvested and frozen at –20° C or tested immediately in some cases following centrifugation. Fresh whole blood with and without anticoagulant (lithium heparin) were stored at room temperature (20 °C) and shipped immediately upon collection to the Canadian Food Inspection Agency, Mycobacterial Diseases Centre of Expertise (MDCE), Ottawa, Ontario for evaluation using the LST and FPA respectively.

3.2.2 Cervid TB Stat-Pak™

This test (Cervid TB Stat-Pak Assay™, Chembio Diagnostic Systems, Inc.) employs a cocktail of selected *M. bovis* antigens including ESAT-6, CFP10, and MPB83. The assay was performed as described previously (Lyashchenko et al., 2008). Briefly, 30 µL of serum was placed in the samples well, followed by adding three drops of running buffer. The test was read visually 20 minutes later. Any visible band in the test area was considered an antibody positive result, whereas no band was recorded as a negative result.

3.2.3 Lymphocyte Stimulation Test (LST)

The LST was performed as described by (Surujballi et al., 2009). Briefly, peripheral blood mononuclear cells (PBMCs) were extracted from blood collected in lithium heparin glass vials and stored at/ near room temperature for 24 to 48 hours. For each animal the PBMCs were

divided into 24 wells in a 96 well tissue culture plate and exposed to four antigens, a positive control and a negative control each in quadruplicate wells. The four test antigens included the current *M. bovis* PPD tuberculin Canadian standard preparation and *M. bovis*, *M. avium ssp avium* and *M. avium ssp paratuberculosis* PPD tuberculins. All PPD tuberculins were obtained from the Biologics Production Unit of the MDCE, CFIA. Concanavalin A was used as a positive control to monitor lymphocyte viability and the medium was used as the negative control. The plates were incubated at 37ºC in 5% CO2 for 4 d, after which 0.4 μCi of [3H]-thymidine was added to each well and the plates reincubated as before for an additional 8 h. The [3H]-thymidine uptake was measured in counts per minute (cpm) with a Matrix 96 Direct Beta Counter (Packard Instrument Company, Meriden, Connecticut, USA). A sample was scored *M. bovis* positive if the Δcpm value (mean cpm of the antigen minus mean cpm of the negative control) for either of the *M. bovis* tuberculins was >= 5000 and the Δcpm value of each of the avium and the paratuberculosis antigens was < 66% of that of the higher *M. bovis* Δcpm value. Samples were classified as mycobacteriosis positive if the Δcpm value for at least one of the *M. bovis* antigens was >=5000 and the Δcpm value of the avium or paratuberculosis antigen was > 66% of the higher bovis value. A sample was scored negative if the Δcpm values for the *M. bovis* antigens were less than 5,000. A sample was declared unfit if there was no stimulation with concanavalin A. A classification of mycobacteriosis was considered a negative result for this assay as other cross-reacting mycobacteria such as *M. avium* and *M. avium paratuberculosis* will result in a positive interpretation.

3.2.4 Fluorescence Polarization Assay (FPA)

The FPA, utilizing a fluorescein-labelled MPB70 protein antigen was performed following the method described by Surujballi et al (2002). Briefly, serum was diluted 1:5 (final volume 1 mL) in PBS (0.01 M phosphate and 0.85% sodium chloride, pH 7.4) supplemented

with sodium azide (0.1%) and lithium dodecyl sulfate (0.1%). After equilibration at room temperature for 1 hour, a blank reading was taken in a fluorescence polarization analyzer (Sentry model; Diachemix, Grayslake, Illinois, USA). An aliquot of fluorescein-labelled MPB70 protein was then added and the mixture vortexed. After equilibration for 30 min at room temperature, the blank-subtracted fluorescence millipolarization (mP) value was obtained and compared with the mean of the blank-subtracted mP values of 3 serum samples from elk known to be negative for bovine tuberculosis that were included in every assay (negative controls, from a prior experimental infection study). A Δ mP value (mP value of the sample minus mean mP value of the negative controls) of less than 10 was scored as negative. A value of 10 to less than 15 was scored as suspicious; a value of 15 or greater was scored as positive. A serum sample from an elk infected with *M. bovis* was included in every assay as a positive control. Both positive and suspicious samples were considered positive for the purposes of this study.

3.2.5 Sampling methodology

Elk or deer testing positive (parallel interpretation) on any one of these three tests (FPA, LST, Cervid TB Stat-Pak) were subsequently recaptured from 1 day to 3 months later using the same methodology described above, euthanized with a captive bolt gun and slung by helicopter to a central laboratory for immediate necropsy and cultured for mycobacteria as described below. Elk or deer testing negative on all three of the tests were not recaptured, but were monitored by aerial telemetry until their radio collars fell off within 3-12 months after capture. A subset of 173 elk and 80 deer that were culled for population control measures (n=124 elk, n= 76 deer) or that died during initial capture due to cervical fractures (n=49 elk, n=4 deer), were also used as part of a validation subsample as these animals were not removed based on *a priori* blood testing results (Figure 3.2).

For elk and deer that were culled or euthanized due to a positive blood test, multiple tissues were collected at necropsy as part of a detailed post-mortem collection procedure that was very thorough. This is comparable to other studies involving European badgers (*Meles meles*) where detailed necropsy procedures were used (Chambers et al., 2008). Peripheral lymphoid tissues examined and collected were submandibular, medial and lateral retropharyngeal, parotid, palatine tonsil (tonsillar crypt), prescapular, popliteal, prefemoral, supramammary/testicular, internal iliac, hepatic, portal, mesenteric, bronchial, and mediastinal lymph nodes. Pooled tissue from body, head, abdominal and thoracic lymph nodes were submitted for mycobacterial culture, histopathogical examination and acid-fast staining, only when evidence of gross visible lesions were observed at necropsy. Initially, all lymphoid tissue was submitted for culture and histopathological examination from all necropsied elk and deer, but after 2006, only tissue with gross visible lesions were submitted for culture and histopathology, as all elk and deer with positive cultures also had visible lesions (unpublished data). All other major organ systems were systematically examined for gross lesions indicative of mycobacteriosis and any suspect tissue was also sent for mycobacterial culture, histopathological evaluation, and PCR testing to confirm identity of cultured mycobacteria (Lutze-Wallace et al., 2005b).

Harvested tissues were either frozen at –20° C or refrigerated and were shipped to the MDCE in Nepean, Ontario within 24 to 48 hours of collection. Formalin-fixed tissues were embedded in paraffin, cut into sections 5 mm thick, and stained with hematoxylin and eosin as well as by the Ziehl–Neelsen technique for detection of acid-fast bacilli. Slides of the tissue sections were examined by a pathologist experienced in the diagnosis of *M. bovis*. The tissues were cultured for mycobacteria using the method described by (Rohonczy et al., 1996). Inoculated media were incubated at 37° C for 12 weeks and examined every 2 weeks for

evidence of bacterial growth. Culture isolates were confirmed as *M. bovis* using a PCR assay for the IS6110 using a 123 base pair amplicon for identifying *M. tuberculosis* complex mycobacteria according to (Miller et al., 1997). Elk were considered positive on the second stage of this sampling if *M. bovis* was cultured from any tissue at necropsy. Personnel conducting culture isolation were occasionally aware of prior blood test results from some animals, but this did not influence whether or not culture results were considered positive or negative and did not change how cultures were evaluated. Lesion severity scores were assigned at necropsy based on those described for red deer by Nugent (2005).

3.2.6 Statistical analyses

3.2.6.1 Classical two-stage analysis

A classical two-stage analysis utilizing parallel interpretation of all three tests for the first stage and using detailed necropsy and mycobacterial culture as a gold standard (Dohoo et al., 2010) was carried out on all elk and deer which were euthanized because of prior positive test result on one of the three tests. Two separate analyses were conducted using this dataset; one that assumed that all serially test negative elk and deer (negative on all three tests) were true disease negatives (uncorrected classical two-stage analysis), and a second analysis (bias corrected classical two-stage analysis) that corrected for the potential verification bias introduced by sampling primarily test positive elk and deer, as very few of the test negative animals were validated using the gold standard test (Greiner and Gardner, 2000).

3.2.6.2 Validation subsample

Elk or deer that died during capture or were intentionally culled were also used for a separate validation subsample analysis using classical techniques, but with only a single stage of necropsy and culture, (Dohoo et al., 2010) as they were sampled independently of prior blood testing and blood testing was carried out immediately post-mortem (within 5 minutes) using the

same techniques or from blood sampled just prior to euthanasia for culled animals. These analyses all assume conditional independence between all three tests.

3.2.6.3 Bayesian latent class analysis

The Bayesian latent class model for test validation was implemented using WinBUGS version 1.4.3 using previously described methodology (Branscum et al., 2005; Toft et al., 2007; Drewe et al., 2009) for a two-population sample. This model estimates test parameters in the absence of a gold standard assuming a latent variable (disease status) and was used to ensure that the final model was identifiable (Branscum et al., 2005; Toft et al., 2007). Two subpopulations of elk and deer were found through initial sampling; a high risk zone termed the Western Control Zone and a low risk population outside this zone (Lees et al., 2003; Shury and Bergeson, 2011). Two different variations of this analysis were run: 1) one where two of the tests are considered conditionally dependent (FPA and Cervid TB Stat-Pak) and the third is conditionally independent (LST, CD model), and, 2) one where all three tests are considered conditionally independent (CID model). This satisfies the major assumptions of latent class models as prevalence of *M. bovis* in both elk and deer varies between these two areas, elk are considered genetically distinct populations (Vander Wal et al., 2012a), and the test characteristics could be assumed to be constant across these two populations. The FPA and Cervid TB Stat-Pak are both humoral antibody tests which measure similar, but different secretory antigens of *M. bovis* (Chambers, 2009; Surujballi et al., 2009), while the LST is a test which measures a cell mediated immune response (Hutchings and Wilson, 1995). Markov Chain Monte Carlo simulations were conducted with a burn-in of 5,000 iterations to allow model convergence. The joint posterior distribution was estimated from 55,000 MCMC iterations, discarding the first 5,000 iterations for burn-in. Marginal posterior distributions for the model parameters were summarized using medians and 95% credible intervals for sensitivity, specificity, positive and negative covariance

values, and prevalence for both species between the two subpopulations. Model convergence was assessed using visual inspection of time series, visual checking of the kernel density plots as well as Gelman-Rubin plots from three chains started with dispersed initial values. Prior distributions for sensitivity and specificity estimates were derived from published literature (Table 3.1) and beta distributions were created from these estimates using BetaBuster 1.0 software (freely available at http://www.epi.ucdavis.edu/diagnostictests/betabuster.html).

A sensitivity analysis was conducted using partially informative prior distributions for prevalence, sensitivity and specificity estimates to determine the sensitivity of the model to prior information. Partially informative uniform priors were used for both sensitivity (0.2, 0.8) and specificity (0.5, 1) for all three tests and a non-informative beta (1,1) prior was used for prevalence. The priors for covariance of sensitivity (g_{Se}) and specificity (g_{Sp}) were given uniform priors with minimum and maximum limits of the distribution (Toft et al., 2007). Flat beta prior distributions modelled on the interval between 0 and 1 for sensitivity and specificity were also explored, but these could not be modelled due to the limits imposed by the covariance maxima and minima (non-convergence and multi-modality).

3.2.6.4 Measures of Test Performance

Percentage agreement between the three tests on elk that tested positive on at least one test or negative on at least one test were also calculated (Drewe et al., 2009) for elk on their initial capture only (live captures plus capture mortalities only). Sensitivity and specificity covariance and kappa values were also calculated for elk and deer sampled in the two-stage sampling using techniques and formulas described in (Gardner et al., 2000). Percent of maximum sensitivity and specificity covariance were also calculated as a measure of test dependence. Predictive values, post-test probabilities of infection, area under the curve (AUC), diagnostic odds ratios given the assumed prevalence in the population (from Bayesian latent class analysis)

were calculated using STATA IC version 12 software using the diagt module (Seed, 2001) for diagnostic test evaluation. All analyses were conducted separately for elk and deer.

3.2.6.5 Seroprevalence

Seroprevalence was assessed for each test separately and in parallel, where all three tests were available for a captured elk or white-tailed deer. Age was estimated based on cementum annuli as described above for animals that were euthanized, but was estimated by incisor wear for elk or deer that were captured and released alive into one of five age categories (<1 year, 1 to 2 years, 3 to 5 years, 6 to 8 years and 9+ years). Serological results were considered positive or negative as described in sections 3.3.2 to 3.3.4. A multivariate logistic regression analysis was completed using the parallel test interpretation for all three tests (LST, FPA and Stat-Pak) as the outcome variable using STATA 13.1. Only animals which had all three test results that were captured between 2004/05 and 2013/14 were used in this analysis and 7 independent variables were screened using univariate logistic regression, with all variables having a p-value less than 0.2 being kept in the multivariate model. The majority of animals captured prior to 2004/05 did not have results for all three tests, thus only animals captured during and after this year were used. The final multivariate logistic model was developed using a backward stepwise selection procedure until all variables were significant at a p-value <0.05.

3.3 Results

Cross tabulated counts of single diagnostic tests and test combinations for the Bayesian and classical two-stage analyses for elk and deer are summarized in Tables 3.2 and 3.3. Thirty-three out of 483 (6.83%) tested elk that were subjected to the gold standard, were culture positive following detailed necropsy from an average of 48 elk that were cultured annually (range 1 to 92) between 2002 and 2011. Only 3 out of 108 (2.78%) tested white-tailed deer that were subjected to the gold standard were culture positive following necropsy from an average of 18

(range 1 to 53) deer cultured annually. Approximately 40% of tested elk were male and 60% were female, while 36% of tested deer were male and 64% of tested deer were female, which was consistent across the two subpopulations but varied on an annual basis (data not shown). More elk and deer were tested in the WCZ compared to outside (78% versus 22%) this zone due to interest in obtaining accurate estimates of prevalence over time and management objectives which required removal of infected elk and deer from this area as part of control programs. A range of disease severity was present in culture positive elk and deer with the majority having moderate to severe lesions as described by (Nugent, 2005). Thirty out of 36 culture positive elk and deer (83.3%) had lesion severity scores greater than or equal to four, while 6 (16.7%) of 36 were three or less (Nugent, 2005).

3.3.1 Classical Two-Stage Analysis

For elk, the LST exhibited the highest diagnostic odds ratios and AUC values while FPA values were lowest for the classical analyses, while these values were highest for the Cervid TB Stat-Pak for the Bayesian analysis and lower for the FPA and LST (Table 3.6). Extremely wide confidence intervals (0.8% to 91%) for the classical two-stage analysis made the sensitivity estimates practically meaningless as only 3 culture positive deer were used (Table 3.8). Specificity estimates for both elk and deer were generally quite high with confidence intervals overlapping for all three tests individually. Negative predictive values for both species approached 100% when tests were interpreted in parallel with corresponding very low values for post-test probability of a negative test (Tables 3.6, 3.8, 3.9).

3.3.2 Bayesian Latent Class Analysis

Model convergence was achieved with both latent class models, but some autocorrelation was present in the chains for FPA and Cervid TB Stat-Pak sensitivity estimates. Monte Carlo

standard errors were all very small compared to mean estimates, indicating high precision of the posterior estimates at the run length used.

Dependence between the FPA and Stat-Pak varied between elk and deer. For elk, the DIC value for the Bayesian conditional dependence model was 89.08 and that for the conditional independence (CID) model was 88.99 indicating lack of covariance (Spiegelhalter et al., 2002), even though the sensitivity covariance (γSe) values from the FPA-Stat-Pak conditional dependence model (γSe) was seemingly low, 0.0145 (-0.094, 0.108) and the 95% CI overlapped zero. The maximum sensitivity covariance was only 16.3%, indicating little or no dependence between these two tests in terms of sensitivity (Gardner et al., 2000). Similarly, specificity covariance (γSp) was 0.002 (-0.002, 0.009), also overlapped zero and was only 5.1% of the maximum indicating little or no dependence with specificity.

In contrast, sensitivity covariance values for Stat-Pak and FPA were moderately high (-0.091) and was 56% of the maximum value (Table 3.5) indicating some dependence between the two tests for deer, unlike elk. Specificity covariance values for deer and elk were very similar and quite low indicating lack of dependence in terms of specificity. Posterior estimates from the Bayesian analysis were very sensitive to prior distributions, particularly the estimates of test sensitivity which varied between 3% and 51.7% from the estimates with prior information (Table 3.7). The sensitivity estimates of the FPA and Cervid TB Stat-Pak differed the most (36.5% and 51.7% respectively), while the sensitivity estimate for LST was not influenced by the prior distribution (3.1%). Specificity estimates, on the other hand were relatively insensitive to the prior distribution and all estimates differed by less than 4%.

3.3.3 Comparison between analyses and test agreement

Test sensitivities varied substantially more than test specificity estimates between the three different analyses for both species, the latter which tended to be relatively similar

regardless of validation methodology (Tables 3.3, 3.8, 3.10 and 3.12), with FPA having the lowest sensitivities and either LST or Cervid TB Stat-Pak having the highest test sensitivity depending on the analysis. Bayesian estimates of test sensitivity were generally higher than the classical two-stage estimates, especially for deer where low numbers for the classical two-stage analysis resulted in imprecise estimates of sensitivity. Uncorrected sensitivities were generally higher compared to bias corrected estimates while specificities were lower.

Sensitivity and specificity covariance values differed substantially between the classical two-stage analysis and the Bayesian analysis (Table 3.5). Prevalence estimates differed for both the WCZ and the area outside the WCZ, dependent on the analysis. The Bayesian prevalence estimate for elk in the WCZ was lower than the corresponding classical estimate, while the Bayesian prevalence estimate for areas outside the WCZ was higher than the classical estimate (Table 3.2). Kernel density estimates for the Bayesian parameters are provided in the Appendix.

All three tests rarely agreed on positive results in elk (2.7%) while the LST was often positive (54.7%) when the other two tests were negative (Figure 3.3). Cervid TB Stat-Pak and FPA agreed on positive tests in elk (6.34%) more frequently than the other two combinations of tests (3.02% and 2.11%). Similar results were observed for deer (data not shown).

3.3.4 Seroprevalence

Parallel seroprevalence decreased significantly over the 10 years of observation, with an 8.4% average annual decrease in odds of testing seropositive (Table 3.14, Figure 3.4). This decrease was most apparent after 2010/11 and was more apparent in the Core Area versus the rest of the GRME (Figures 3.5 & 3.6) for both elk and white-tailed deer. Elk had approximately 2.4 times the odds of testing seropositive compared to deer, and animals inside RMNP had 42.9% lower odds of testing seropositive than animals outside the park. Seropositivity for elk has primarily been on the cell-mediated test (LST) in the past four years, with little or no reactivity on the

antibody based tests (Stat-Pak and FPA) (Figure 3.4). Parallel seroprevalence (positive on at least one of the 3 tests) was approximately 33.6% lower in male elk compared to female elk and this was consistent both inside and outside the Core Area (Figure 3.7) and occurred earlier in male elk which have exhibited no seropositivity in recent years (Figure 3.10). Exposure to *M. bovis* was highest in elk in the 3 to 5 year old age category and decreased in older ages, while in deer it peaked in 1 to 2 year old deer and no seropositive deer were found older than the 3 to 5 age category (Figure 3.8). Seroprevalence differed by age category between sexes for elk with female elk decreasing over time, while seropositivity for males increased with age (Figure 3.9).

3.4 Discussion

3.4.1 Individual Test Performance Characteristics

This study provided a unique opportunity to both compare different test validation approaches and accurately estimate various test parameters both separately and in combination for three blood based assays in two free-ranging cervid species that were naturally infected with *M. bovis*.

All 95% confidence intervals for single test sensitivity analyses overlapped for all three tests for both elk and deer with the exception of the Cervid TB Stat-Pak in elk, for which sensitivity estimates were significantly higher on the Bayesian analysis compared to the bias corrected classical. Bayesian estimates of sensitivity were generally higher than the classical two-stage estimates with LST and Cervid TB Stat-Pak being very similar in elk, and FPA sensitivity being substantially lower. In contrast, for white-tailed deer median sensitivity estimates of FPA and Stat-Pak were very similar, while LST median estimates were higher than both these tests. Specificity estimates differed between validation methods with uncorrected classical estimates generally being lower than the both the Bayesian and bias corrected estimates

of specificity. This is likely a result of uncorrected specificity estimates being considered invalid in an infected population due to a significant proportion of elk likely being *M. bovis* positive with paucibacillary infections, and thus being negative on culture (gold standard test). This is why specificity estimates are typically derived from known disease negative populations, while sensitivity estimates are derived from known infected populations when using gold standard tests for validation studies (Toft et al., 2005). Classical approaches to test validation often overestimate sensitivity, as they assume conditional independence between tests (Gardner et al., 2000; Branscum et al., 2005), which is often not the case. Conversely, Bayesian test validation approaches adequately model dependence between tests, but are very sensitive to the dependence structure being modelled (Albert and Dodd, 2004). These authors recommend using a gold standard when possible, and that conducting gold standard verification (i.e. partial verification or validation subsampling) on a fraction of subjects may aid in choosing a model. Credibility and confidence intervals for the FPA and LST overlapped for elk due to very different estimates of sensitivity for these two tests for the classical two-stage analysis, indicating that these two tests do vary substantially in their ability to detect M. bovis positive elk. Sensitivity confidence intervals do overlap for FPA and LST in the Bayesian and validation subsample estimates, indicating that the choice of validation method becomes important when comparing tests. Specificity estimates for elk for the Cervid TB Stat-Pak were significantly higher than the LST regardless of validation method, indicating that more false positive elk were identified by the LST than the Cervid TB Stat-Pak. A similar pattern is observed in white-tailed deer, with the exception that significantly lower specificity for the Stat-Pak compared to the FPA is observed in the Bayesian analysis, due to wide overlapping confidence intervals for the validation and classical two-stage estimates. Sensitivity estimates for the three tests are only really valid with

Bayesian validation analysis, as the other two validation methods exhibit such wide confidence intervals that their estimates are not useful for comparison. This is likely due to the gain in power that the Bayesian analysis achieves by using data from a higher number of animals.

The degree of dependence between tests varied substantially between species. Bayesian estimates of sensitivity and specificity covariance in elk failed to indicate any significant covariance between any of the three test combinations (Table 3.5), including FPA and Stat-Pak which were expected to be dependent due to their similar biological basis. All Bayesian covariance estimates were a relatively small percentage of the maximum, and kappa values were correspondingly small for elk. Conversely, sensitivity covariance was moderate to high for the FPA Stat-Pak combination in both the Bayesian and classical two-stage analysis for white-tailed deer, indicating significant dependence between the two tests in this species. The other two test combinations (LST with FPA or Stat-Pak) failed to exhibit significant covariance for either sensitivity or specificity and values were negative indicating lack of positive dependence between these test (Gardner et al., 2000). This makes intuitive sense, as there is a strong biological basis for dependence between the FPA and Cervid TB Stat-Park as both are humoral antibody tests, lending more credibility to the two-stage classical analysis over the Bayesian conditional dependence approach, at least for elk. For white-tailed deer, the FPA and Stat-Park were conditionally dependent and because of lack of data and power in the classical two-stage and validation subsample, the Bayesian estimates for this species are likely the most valid.

Results of this study further validate the recommendations of Albert and Dodd (2004) that gold standard verification be used for test validation purposes whenever possible, even if the gold standard is imperfect or can only be done for a fraction of subjects. This is likely especially true for diagnostic testing strategies in wildlife populations, where logistical difficulties and costs

involved in capturing and testing large numbers of animals prohibits large scale validation using gold standard tests. This is not the case though where very small numbers of animals are available for gold standard verification (e.g. 3 *M. bovis* positive white-tailed deer in this study), and Bayesian validation methods are preferable, due to their ability to gain power from larger numbers of animals.

The sensitivity of both the FPA and Cervid TB Stat-Pak to the prior values used in the Bayesian latent class analysis indicates a lack of statistical information in the data. This may be due to a relatively small difference in apparent prevalence between the western control zone and areas outside (Table 3.2). This could be a result of the antibody based tests detecting elk that are either latently infected or been exposed to *M. bovis* and not developed serious clinical disease, resulting in similar prevalence estimates between the two risk areas.

3.4.2 Optimal test combinations

Optimal sensitivity was achieved with just two of the three tests in elk; the LST and Cervid TB Stat-Pak in combination when interpreted in parallel, resulting in very high sensitivity as well as the highest diagnostic odds ratio and area under the curve (AUC). Since the surveillance goal in this program was to detect as many *M. bov*is infected elk as possible for removal, sensitivity was the most critical element of surveillance, even though this results in many false positive tests due to the corresponding loss of specificity. Other *M. bovis* testing regimes in wildlife reservoirs may have other goals, especially if highly valued or endangered species are involved, where high specificity would be the ultimate goal to reduce the number of false positives. Some additional diagnostic value is achieved with the addition of the FPA to the LST and Cervid Stat-Pak, as diagnostic odds ratio and post-test probability of a positive test does increase when all three tests are interpreted in parallel. But, if the cost of this test was high or could not be performed easily, it would not provide additional diagnostic value. Conversely, the

LST is a difficult test to perform in field situations due to requirements to ship samples to a specialized lab within 24 to 36 hours of collection and maintain samples at room temperature. This resulted in approximately 10% of all blood samples being unfit for LST testing by arrival at the laboratory for this project, despite being relatively accessible compared to other more remote areas of North America. If one considers the validation subsample the least biased methodology, the Cervid TB Stat-Pak had the best combination of sensitivity and specificity of all three tests in elk when interpreted as a single test in isolation based on AUC values, although the FPA had higher DOR than the other two tests using this methodology.

Due to the issues with low numbers and wide confidence intervals for classical two-stage and validation subsample methods in white-tailed deer, the Bayesian estimates likely provide the most valid estimates for determination of optimal combination. The Stat-Pak and FPA combination had the best diagnostic odds ratio and AUC for deer, mostly due to negative sensitivity covariance which boosted the sensitivity of this combination.

The parallel blood testing strategy employed for this test and removal strategy maximized the sensitivity of the screening test, with overall test sensitivity of the parallel test ranging from 95.6% to 100% for elk and 92.3% to 100% for deer, depending on the validation method. This created a corresponding reduction in specificity resulting in numerous false positives on initial screening. This was felt to be a necessary part of the test and removal program as reductions in sensitivity would have resulted in false negative animals being released into the population. The high sensitivity and negative predictive value of parallel testing resulted in finding almost all bTB positive animals of both species. This boost in sensitivity was achieved in similar fashion with only the Cervid TB Stat-Pak and LST in combination for elk, without the addition of the FPA. The second stage of sampling was the application of detailed necropsy and culture to blood

test positive elk and deer. Mycobacterial culture at necropsy has high specificity and when applied serially after blood testing resulted in very low post-test probabilities of infection for a negative test (0 to 0.003 for elk, 0 to 0.001 for deer). The value of this testing regime became important as the main focus of infection was inside a national park, where standard methods of wildlife population control through hunting cannot be utilized. This allowed highly focused targeting of potentially infectious elk and deer without more 'arbitrary' methods such as hunting and culling which do not have a first and second stage to increase the efficiency of testing.

The best validation method for comparing the three tests is likely to be the validation subsample in elk, as it the least biased estimate, but is more likely the Bayesian method for white-tailed deer due to miniscule numbers of *M. bovis* positive deer in the sample. Sensitivity estimates from the validation subsample for both species have relatively wide confidence intervals due to low numbers of positives, making comparisons between tests all but impossible, but specificity estimates had much narrower confidence intervals overall due to larger numbers of animals sampled. The bias corrected classical two-stage estimates of sensitivity and specificity for elk were generally quite close to the validation subsample estimates with the exception of the Cervid TB Stat-Pak, for which the uncorrected sensitivity and specificity estimates were closer to the validation subsample estimate.

Even though the validation subsample analysis was conducted on a relatively small number of elk (n=173) and smaller number of deer (n=80), the estimates were very similar to those obtained using the classical approach on a larger sample. The sampling fraction of test positives (positive on at least one screening test) for elk, was 58.7% (196/334), but the sampling fraction of test negatives subjected to gold standard testing was only 7.1% (45/635). This indicates that there was likely some verification bias introduced by only sampling primarily test

positive animals, primarily for the LST and FPA. This may be a result of low sample numbers, especially for positive elk in the validation subsample in the sensitivity calculations. This results in the bias corrected sensitivities for the individual tests being substantially lower than the uncorrected calculated values. This may not be the case where detailed post-mortem procedures are not used and only certain targeted tissues such as head lymph nodes are collected for *M. bovis* culture. In these cases, estimates of test performance may be highly biased due to many blood test negative animals being truly disease positive, but false positive on the gold standard test. These are most often early infections which are culture negative and contain few bacterial organisms (paucibacillary) or are latently infected animals (Crawshaw et al., 2008; Gavier-Widen et al., 2009) . As many as 30% of some wildlife species including red deer may not have grossly visible lesions of *M. bovis*, but still be culture positive (Chambers, 2009; Gavier-Widen et al., 2009). These animals may not have a significant role to play in ongoing transmission though, as they are very likely early infections or latent infections, which have little impact when prevalence is low. This population is somewhat different as only 1 elk out of 33 (3%)) culture positive elk and 0 out of 3 (0%) white-tailed deer did not have grossly visible lesions of *M. bovis* at necropsy, but this is due to a detailed post-mortem procedure being used specifically tailored to finding *M. bovis* infection.

3.4.3 Seroprevalence

Overall seroprevalence in both elk and deer has been decreasing in the GRME since 2004/05, and has been on a very steep decline since 2010/11, especially within the Core Area, concurrent with a decline in culture positive animals (Chapter 4). This decline appears to have occurred more rapidly in male elk compared to female elk, with female elk more likely to have been exposed to *M. bovis* than male and elk more likely to have been exposed than deer. The lack of seropositivity on the antibody based tests (FPA & Stat-Pak) also appears to have occurred earlier

in time, with most seropositive elk in the last three years being almost exclusively positive on the cell-mediated test (LST). This likely indicates that these are false positives, based on necropsy and culture results, as none of the animals with exclusively LST positive results (without being positive on other tests) have been positive on culture at necropsy. It may also indicate that elk with early infectious stages are being detected where a type-2 antibody based response has not developed. It would appear that animals with this type of response are very rare or non-existent in this population in recent years. The one culture positive elk that was detected in 2013/14 and the only animal detected in the last three years was positive on the Cervid TB Stat-Pak only, and was negative on the LST and FPA. Infected cervids typically demonstrate strong, early CMI responses in experimental infection with relatively early (compared to cattle) antibody mediated immunity (AMI) appearing at or near the same time (Griffin et al., 2006; Harrington et al., 2008a), although there is marked individual variation in these responses. Female elk were more likely to be seropositive since 2004/05 than male elk and elk were more likely to be seropositive than deer. Surprisingly, animals inside RMNP were less likely to be seropositive, but this was likely to due to the large discrepancy between species within the Core Area (Figure 3.11). If seropositivity equates with exposure to *M. bovis*, exposure is decreasing within this ecosystem and has likely decreased due to management factors which have reduced density of cervids and reduced contact with infected cattle.

3.5 Summary

This study demonstrates that blood tests used for diagnosis of *M. bovis* infections in wild cervid populations compare favourably to currently used skin tests in terms of test sensitivity and specificity, and allow efficient testing of wild populations with a single capture event. These tests offer a significant advantage over skin testing, as a result of only requiring a single handling event. Other studies have determined that blood testing in wild white-tailed deer with a single

test is not very cost efficient in low prevalence populations, although sensitivities were similar to this study (O'Brien et al., 2009). This study benefited from the use of three blood tests in parallel to maximize the number of infected elk and deer being detected on initial screening. All three tests achieved what would be considered relatively moderate AUC values for ROC curves, indicating that they could be used in isolation, but values of AUC improved dramatically when two or more tests were interpreted in parallel. Sensitivity estimates for the currently used tuberculin skin tests range from 80% to 100% in farmed elk and red deer with specificities ranging from 61% to 100% (Cousins and Florisson, 2005). When used individually, only the LST approached levels of sensitivity that would be useful as a stand-alone screening test for *M. bovis* in both elk and deer. The Cervid TB Stat-Pak test has recently been licensed as a screening test in farmed deer and cervid species in the US. Other studies in wildlife reservoirs have demonstrated that, for optimal test performance, multiple diagnostic tests should be utilized in series or parallel for accurate diagnosis of *M. bovis* in many species (Cousins and Florisson, 2005; Chambers, 2009), depending on the objectives of the disease management program. This study validates those conclusions, as combination of humoral and cell mediated tests performed much better than any one individual test in elk, although the two humoral antibody tests performed optimally in white-tailed deer. Test performance of the LST and Cervid TB Stat-Pak in parallel was similar to the use of all three tests in parallel in elk, and so inclusion of the FPA does not greatly enhance test performance, but in deer, the performance of Stat-Pak and FPA in parallel was superior to other test combinations. Period prevalence in a high risk area of southern Manitoba was estimated to be 9.1% in free-ranging *M. bovis* infected elk, while outside this area, prevalence was only 0.76% between 2002 and 2011. Period prevalence in white-tailed deer was much lower, estimated to be 1.15% in the high risk Western Control Zone and 0.12% outside this

area. Blood testing and removal appears to have been a successful strategy to reduce prevalence in this population within parks and protected areas and is one of several factors that are leading towards successful eradication of *M. bovis* from this ecosystem (Shury and Bergeson, 2011).

Seroprevalence is decreasing in this ecosystem over time on all three tests individually and interpreted in parallel for both elk and deer. This decrease occurred earlier for male elk and exposure to *M. bovis* appears to peak when elk are approximately 3 to 5 years of age. Parallel seropositivity was predicted by year of sampling, sex, species and subzone whether or not animals were captured in RMNP, with elk being more likely to be seropositive than deer and females being more likely to be seropositive than males.

Wildlife managers should consider incorporating diagnostic test validation strategies that validate newer blood based assays against gold standards including culture of *M. bovis* from tissues at necropsy where possible, as these data do not exist for many species or is not well validated. Latent class analysis is a reasonable alternative when validation against a gold standard does not exist or cannot be collected for ethical or logistical reasons or sample sizes are too small for precise estimates, but is likely not valid when a reasonable gold standard test such as mycobacterial culture on post-mortem tissues does exist.

3.6 References

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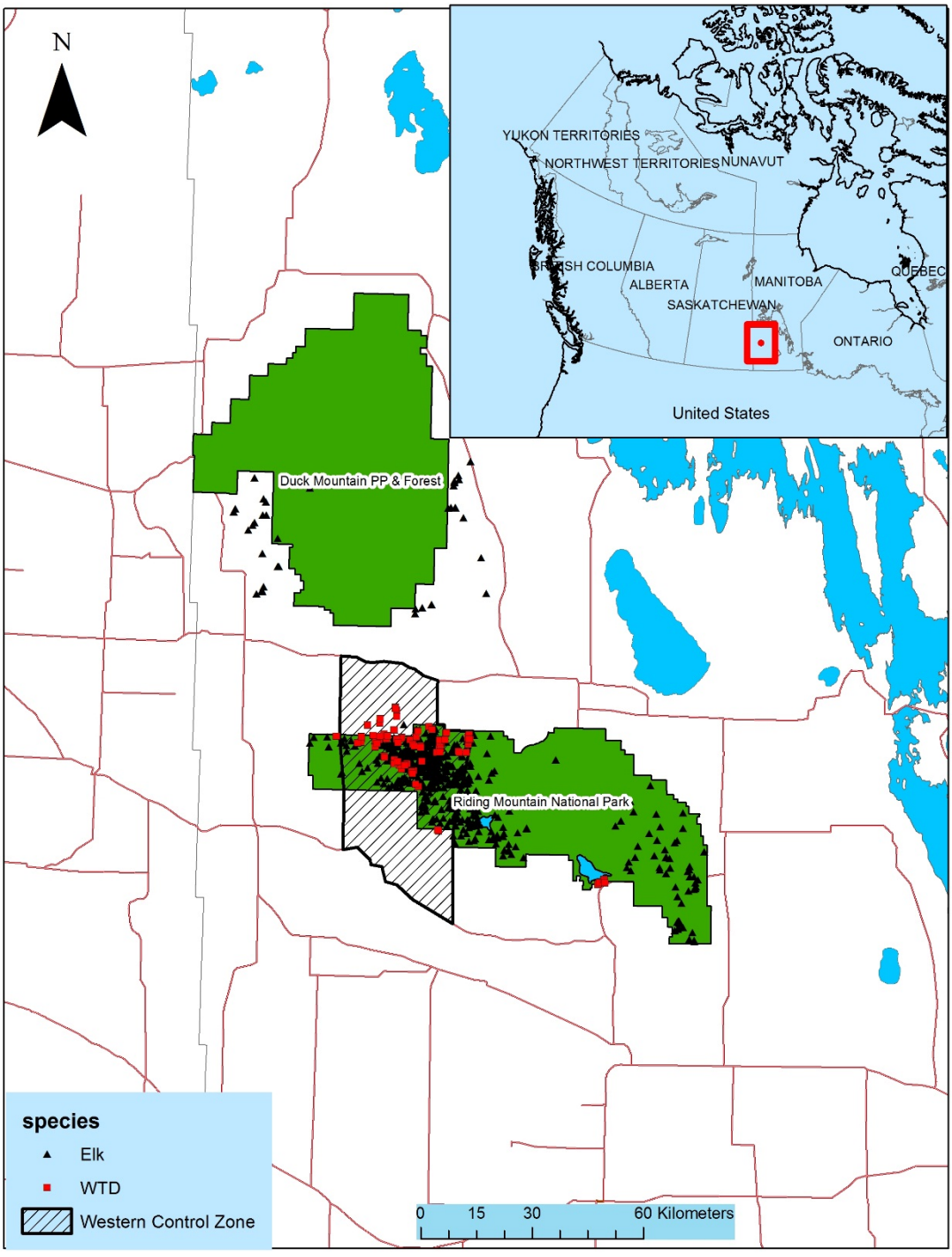

Figure 3.1 Capture locations of free-ranging elk and white-tailed deer in the Western Control Zone and Greater Riding Mountain Ecosystem (GRME) from 2002 to 2012.

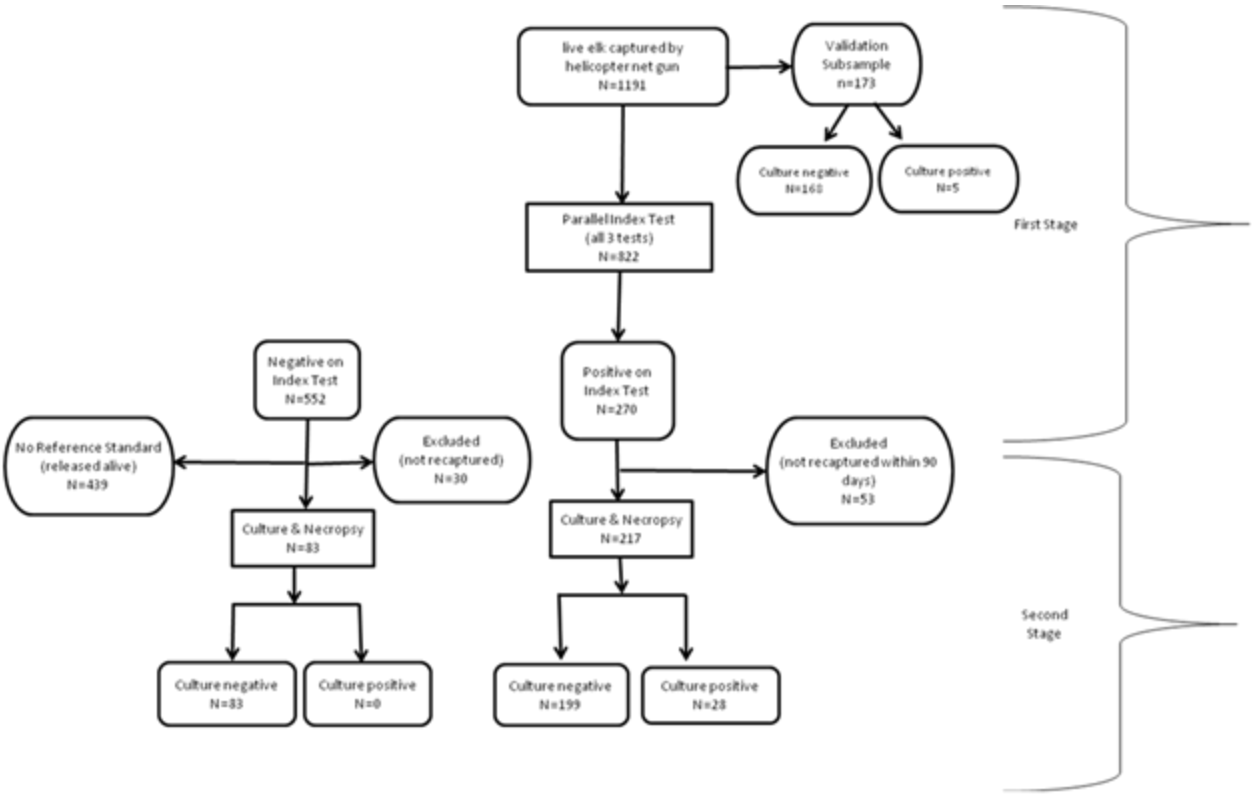

Figure 3.2 Testing algorithm and numbers of wild elk tested in two subpopulations in Riding Mountain National Park between 2002 and 2011.

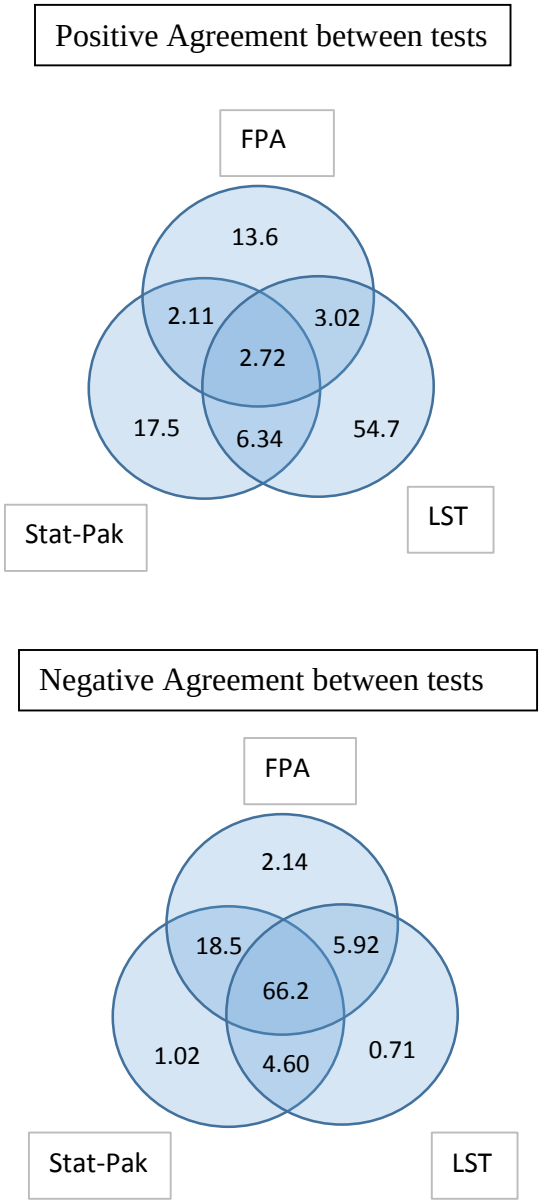

Figure 3.3 Percentage agreement and overlap between the three blood tests on elk captured on initial capture only (not including follow up capture)

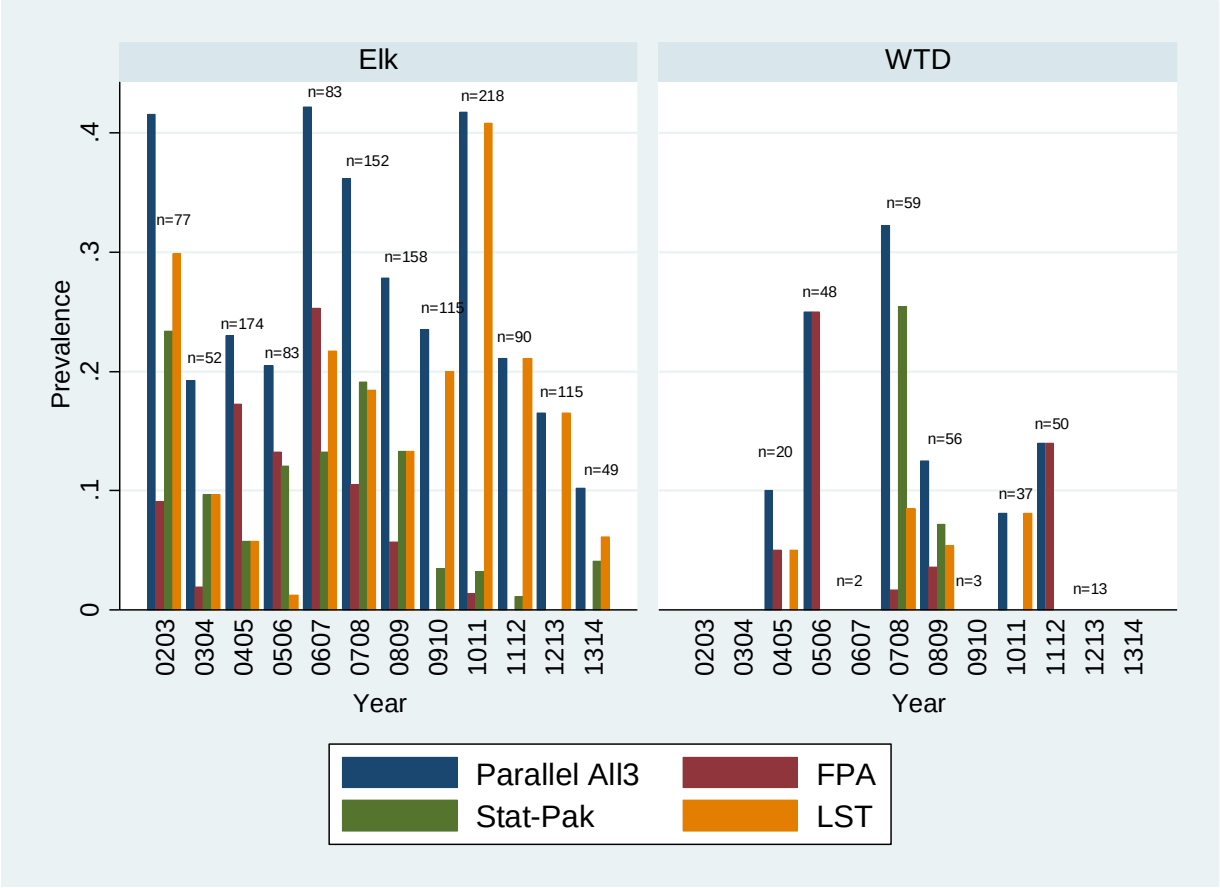

Figure 3.4 Mean annual seroprevalence of captured elk and white-tailed in the GRME for three blood-based assays separately and in parallel by sampling year (biological year from June 1st to May 30th) since 2002.

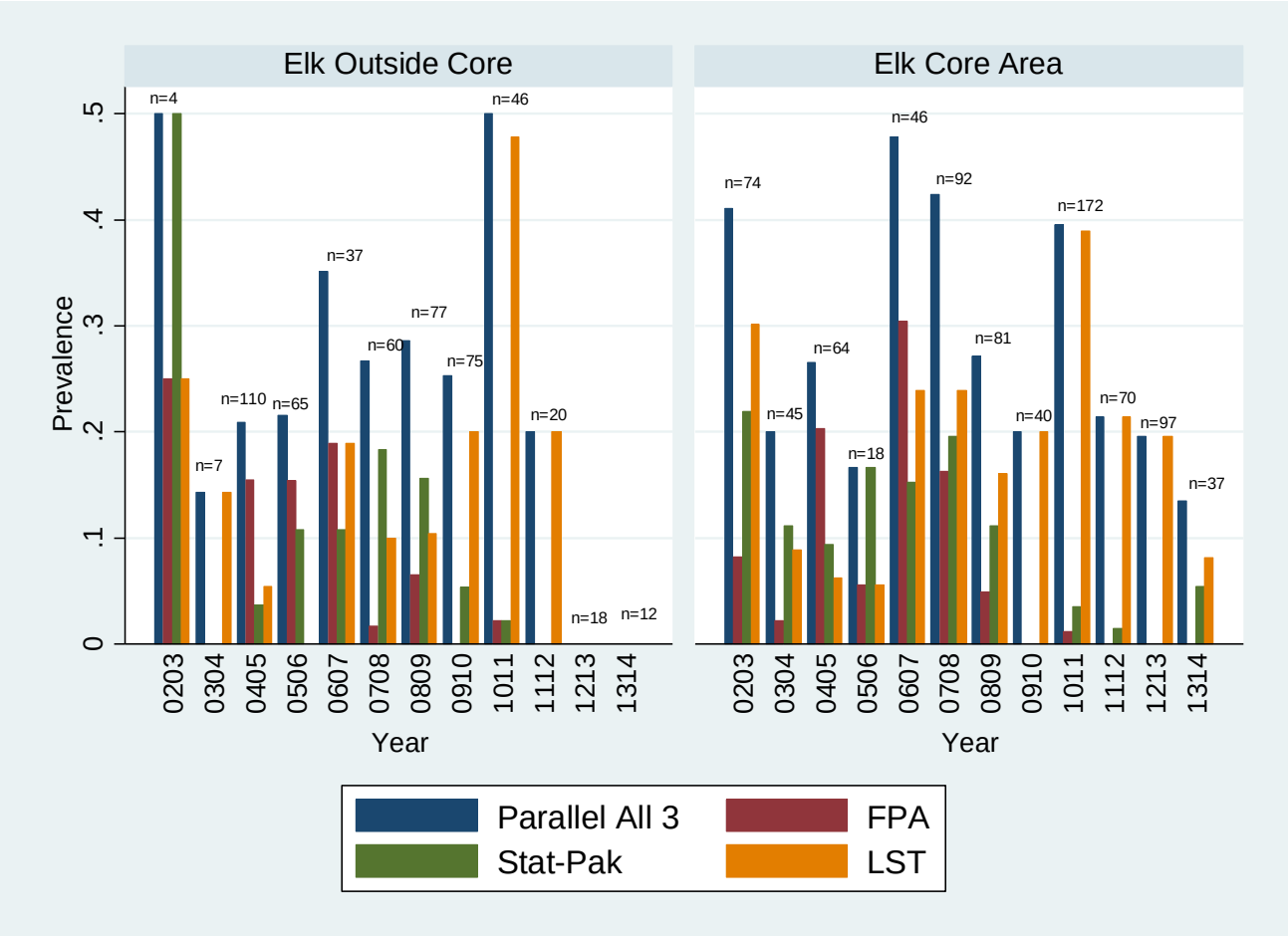

Figure 3.5 Mean annual seroprevalence of captured elk in the GRME for three blood-based assays separately and in parallel by sampling year (biological year from June 1st to May 30th) since 2002 in the Core Area and outside the Core Area.

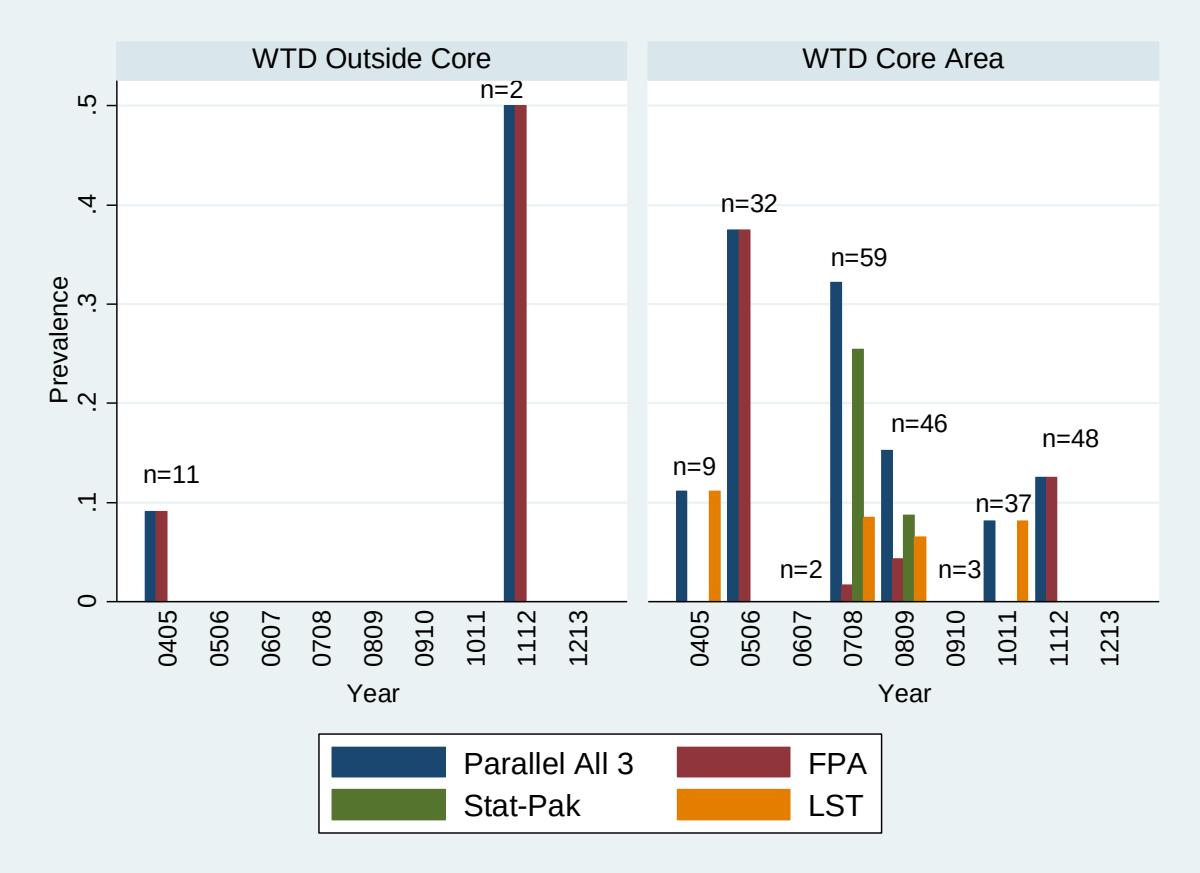

Figure 3.6 Mean annual seroprevalence of captured white-tailed deer in the GRME for three blood-based assays separately and in parallel by sampling year (biological year from June 1st to May 30th) since 2002 in the Core Area and outside the Core Area.

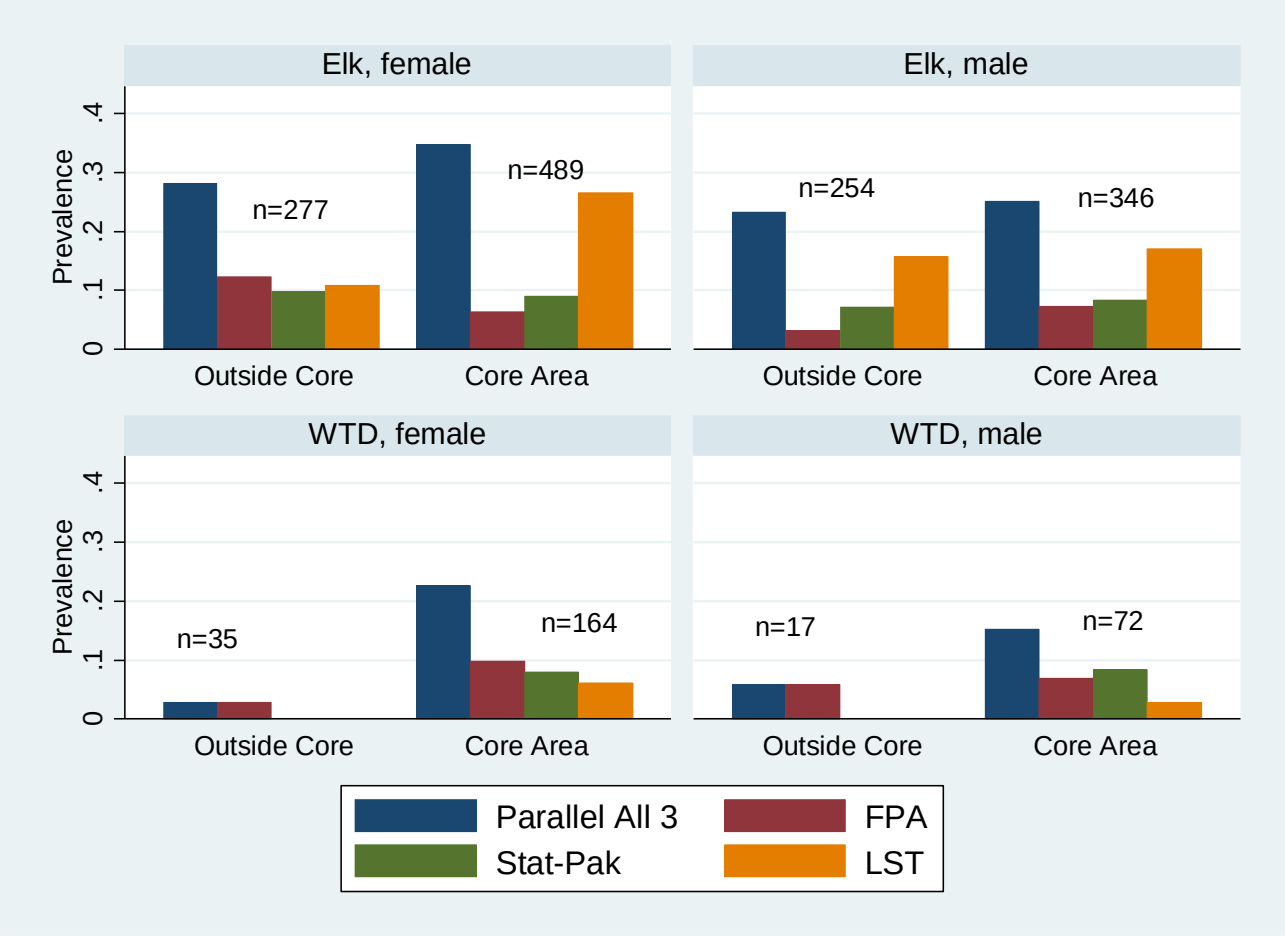

Figure 3.7 Mean seroprevalence of captured white-tailed deer and elk inside and outside the Core Area for each sex for three blood-based assays separately and in parallel since 2002.

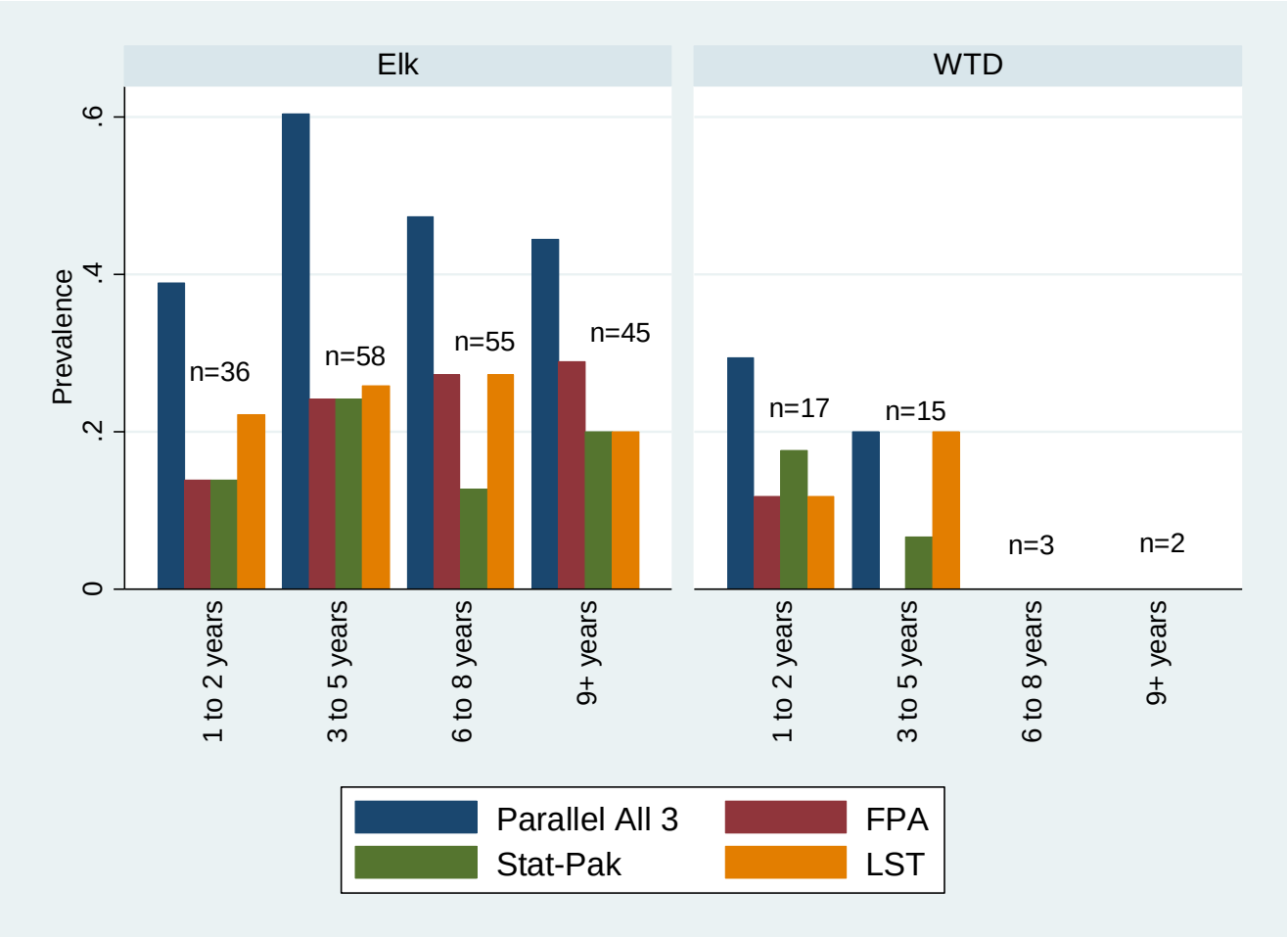

Figure 3.8 Mean seroprevalence of captured white-tailed deer and elk by age category for three blood-based assays separately and in parallel since 2002 in the GRME.

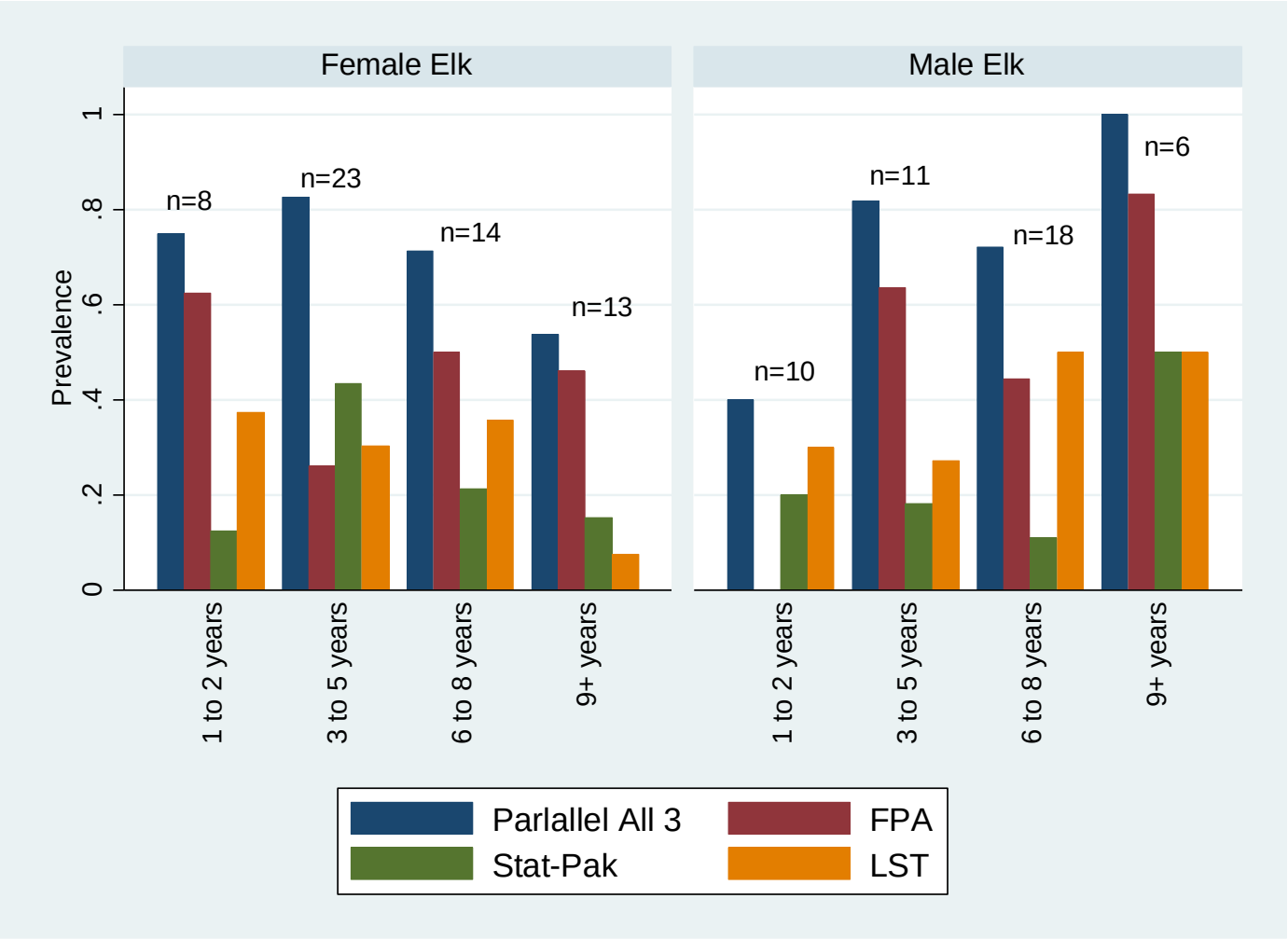

Figure 3.9 Mean seroprevalence for male and female elk captured using three blood-based assays separately and in parallel since 2002 by age category in the GRME.

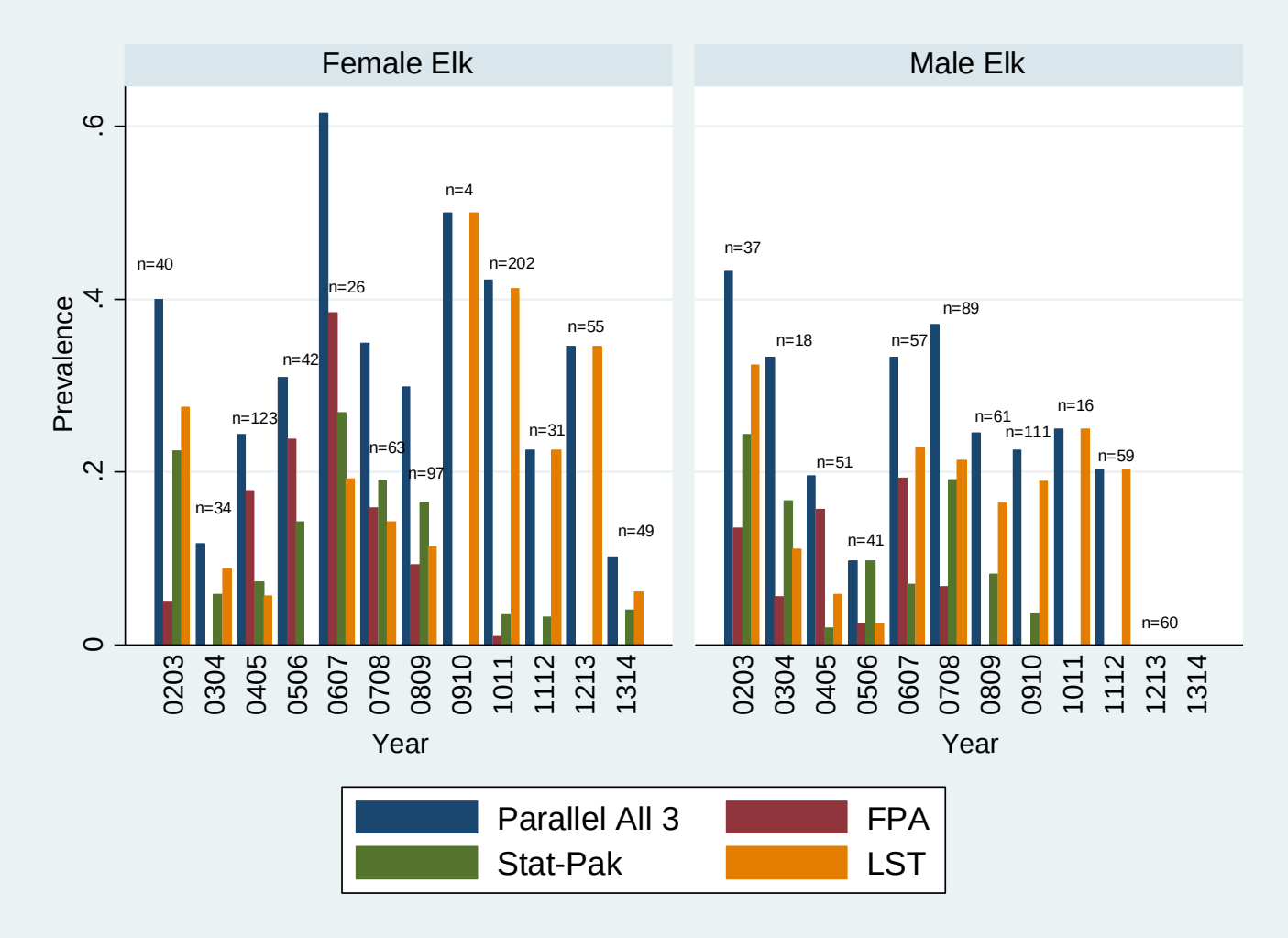

Figure 3.10 Mean annual seroprevalence for male and female elk captured using three blood-based assays separately and in parallel by sampling year (biological year from June 1st to May 30th) since 2002 in the GRME.

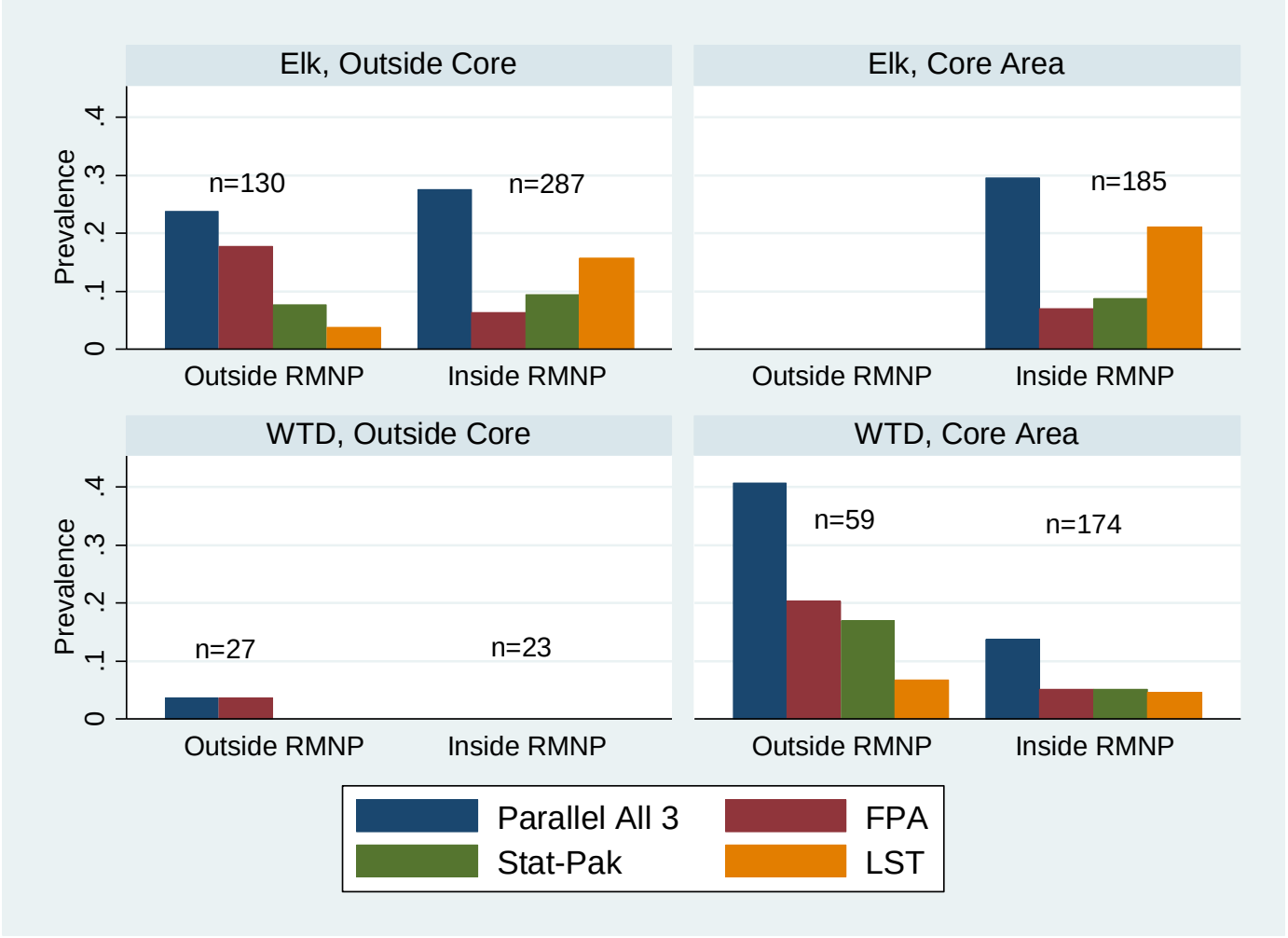

Figure 3.11 Mean seroprevalence for elk and white-tailed deer captured within and outside RMNP in and out of the Core Area for three blood-based assays separately and in parallel since 2002.

Table 3.1 Prior distributions of parameter estimates for latent class analysis of test results from free-ranging elk potentially infected with *M. bovis* in southern Manitoba.

	Diagnostic Test		Mode	2.5^{th}, 97.5^{th} percentile	β distribution (α,β)	Source
WTD	FPA[a]	Sensitivity	0.57	0.24,0.85	4.79, 3.86	Composite sensitivity estimates for elk (mean) from (Harrington et al., 2008b) and (Surujballi et al., 2009). No published WTD data.
		Specificity	0.90	0.78, 0.96	42.57, 5.62	Composite specificity estimate calculated for elk from (Surujballi et al., 2009). No published WTD data.
	Stat-Pak[b]	Sensitivity	56.0	35.3 , 75.0		WTD sensitivity Stat-Pak (O'Brien et al., 2009)
		Specificity	98.9	97.4 , 99.5		WTD specificity Stat-Pak (O'Brien et al., 2009)
	LST[c]	Sensitivity	0.76	0.60, 0.87	30.5, 10.32	Sensitivity estimate for elk from (Hutchings and Wilson, 1995) for LST. Limited published data available.
		Specificity	0.77	0.71, 0.82	172.3, 52.18	Specificity estimate for elk from (Hutchings and Wilson, 1995) for LST. Limited published data available.
Elk	FPA[a]	Sensitivity	0.57	0.24,0.85	4.79, 3.86	Composite sensitivity estimates (mean) from (Harrington et al., 2008b) and (Surujballi et al., 2009)
		Specificity	0.90	0.78, 0.96	42.57, 5.62	Composite specificity estimate calculated from (Surujballi et al., 2009)
	Stat-Pak[b]	Sensitivity	0.75	0.51, 0.89	13.98, 5.33	Sensitivity estimate for deer species from Lyashchenko et al 2008
		Specificity	0.99	0.97, 0.996	272.5, 4.02	Specificity estimate for deer species from Lyashchenko et al 2008
	LST[c]	Sensitivity	0.76	0.60, 0.87	30.5, 10.32	Sensitivity estimate from (Hutchings and Wilson, 1995) for LST
		Specificity	0.77	0.71, 0.82	172.3, 52.18	Specificity estimate from (Hutchings and Wilson, 1995) for LST

[a] – Fluorescence polarization assay
[b] – Cervid TB Stat-Pak
[c] – Lymphocyte stimulation test

Table 3.2 Cross tabulated counts for combinations of three diagnostic tests for diagnosis of *M. bovis* in free-ranging elk from two subpopulations in southern Manitoba.

	LCA (Bayesian)			Classical Two-Stage			
				High Risk Area (WCZ)		Low Risk Area (Outside)	
Test Combination	High Risk Area (WCZ)	Low Risk Area (Outside)	Test Combination	Culture+[a]	Culture-[b]	Culture+	Culture-
FPA[c]+,SP[d] + ,LST[e]+	8	1	FPA+	12	18	0	38
FPA+,SP + ,LST-	3	4	FPA-	38	79	0	79
FPA+,SP - ,LST+	7	3	SP+	13	8	1	15
FPA+,SP - ,LST-	24	21	SP-	30	215	0	75
FPA-,SP + ,LST+	19	2	LST+	25	5	1	37
FPA-,SP + ,LST-	41	17	LST-	107	174	0	79
FPA-,SP - ,LST+	138	43	FPA+, or SP+	19	6	1	48
FPA-,SP - ,LST-	406	242	FPA-, SP-	64	188	0	52
Total tested	646	333	LST+ or SP+	28	1	1	49
Estimated Prevalence (% + 95% CI)	5.57 (2.76, 9.85)	1.57 (0.12, 5.21)	LST-, SP-	124	128	0	52
			FPA+ or LST+	27	3	1	69
			FPA-, LST-	137	147	0	47
			FPA+ or LST+ or SP+	30	0	1	77
			FPA-, LST-, SP-	152	109	0	32
			Total tested	32	319	1	131
			Estimated Prevalence (% + 95% CI)	9.12 (6.09,12.1)		0.76 (0, 2.26)	

[a] – indicates whether elk were culture positive or negative on necropsy.
[b] – Latent Class Analysis
[c] – Fluorescence polarization assay
[d] – Cervid TB Stat-Pak
[e] – Lymphocyte stimulation test

Table 3.3 Cross tabulated counts for combinations of three diagnostic tests for diagnosis of *M. bovis* in free-ranging white-tailed deer from two subpopulations in southern Manitoba.

LCA (Bayesian)			Classical Two-Stage				
				High Risk Area (WCZ)		Low Risk Area (Outside)	
Test Combination	High Risk Area (WCZ)	Low Risk Area (Outside)	Test Combination	Culture Pos[a]	Culture Neg[b]	Culture Pos	Culture Neg
FPA[c]+,SP[d] + ,LST[e]+	1	0	FPA+	1	12	0	4
FPA+,SP + ,LST-	0	0	FPA-	2	82	0	0
FPA+,SP - ,LST+	0	0	SP+	1	8	0	0
FPA+,SP - ,LST-	7	8	SP-	2	86	0	4
FPA-,SP + ,LST+	2	0	LST+	3	8	0	0
FPA-,SP + ,LST-	16	0	LST-	0	79	0	4
FPA-,SP - ,LST+	9	0	FPA+, or SP+	1	20	0	4
FPA-,SP - ,LST-	146	35	FPA-, SP-	2	73	0	0
Total tested	181	43	LST+ or SP+	3	14	0	0
Estimated Prevalence (% + 95% CI)	1.63 (0.53,	0.12 (0.001,	LST-, SP-	0	73	0	4
			FPA+ or LST+	3	20	0	4
			FPA-, LST-		68	0	0
			FPA+ or LST+ or SP+	3	26	0	4
			FPA-, LST-, SP-	0	62	0	0
			Total tested	3	99	0	6
			Estimated Prevalence (% + 95% CI)	2.94 (1.0,8.29)		0 (0, 46.5)	

[a] – indicates whether deer were culture positive or negative on necropsy.
[b] – Latent Class Analysis
[c] – Fluorescence polarization assay
[d] – Cervid TB Stat-Pak
[e] – Lymphocyte stimulation test

Table 3.4 Single test sensitivity and specificity estimates from classical two-stage, Bayesian and validation subsample analysis of free-ranging elk from southern Manitoba.

			Sensitivity	Specificity
			Mean (95% CI)	Mean (95% CI)
Classical Two-Stage Analysis	FPA	Uncorrected	0.40 (0.22,0.58)	0.81 (0.77,0.85)
		Bias Corrected	0.20 (0.06,0.35)	0.92 (0.89,0.94)
	Stat-Pak	Uncorrected	0.62 (0.41,0.83)	0.87 (0.83,0.90)
		Bias Corrected	0.36 (0.16,0.57)	0.95 (0.92,0.97)
	LST	Uncorrected	0.83 (0.70,0.97)	0.64 (0.59,0.68)
		Bias Corrected	0.61 (0.44,0.79)	0.85 (0.81,0.88)
Validation Subsample	FPA		0.20 (0.05, 0.72)	0.99 (0.97, 1.0)
	Stat-Pak		0.60 (0.15, 0.95)	0.90 (0.84, 0.94)
	LST		0.60 (0.15, 0.95)	0.73 (0.65, 0.80)
			Median (95% CI)	Median (95% CI)
Bayesian Latent Class Analysis	FPA	Conditional Dependence (CD)	0.37 (0.19,0.61)	0.94 (0.92,0.95)
		Conditional Independence (CID)	0.38 (0.21,0.62)	0.94 (0.92,0.95)
	Stat-Pak	Conditional Dependence (CD)	0.76 (0.56,0.91)	0.94 (0.93,0.96)
		Conditional Independence (CID)	0.76 (0.57,0.90)	0.94 (0.93,0.96)
	LST	Conditional Dependence (CD)	0.71 (0.55,0.85)	0.79 (0.76,0.81)
		Conditional Independence (CID)	0.71 (0.55,0.84)	0.79 (0.76,0.81)

[a] – Fluorescence polarization assay
[b] – Cervid TB Stat-Pak
[c] – Lymphocyte stimulation test
[e] – Corrected for proportion of test negative and positive elk verified by the gold standard test (culture)(Greiner and Gardner, 2000).
[f] – Uncorrected using standard cross tabulated calculation of sensitivity and specificity assuming equal proportions of test negative and positive elk verified by gold standard.
[g] – Latent class analysis assuming conditional dependence between FPA and Cervid TB Stat-Pak
[h] – Latent class analysis assuming conditional independence between all three tests

Table 3.5 Covariance values, percentage of maximum covariance and kappa values for sensitivity and specificity for pairwise combinations of tests using Bayesian and classical two-stage analyses for elk and deer.

		Test Combination	Sensitivity Covariance	Percent Maximum Covariance	Kappa	Specificity Covariance	Percent Maximum Covariance	Kappa
Elk	Bayesian	Stat-Pak : FPA	0.012	13.45%	0.497	0.002	3.74%	0.124
		Stat-Pak : LST	0.044	32.91%	0.248	-0.004	7.95%	0.026
		LST : FPA	0.030	26.94%	0.496	-0.003	5.97%	0.020
	Classical Two-Stage	Stat-Pak : FPA	0.050	34.38%	0.189	0.004	3.19%	0.027
		Stat-Pak : LST	-0.025	21.15%	0.117	-0.002	1.75%	0.008
		LST : FPA	0.000	0.00%	0.000	-0.031	26.82%	0.152
WTD	Bayesian	Stat-Pak : FPA	-0.091	56.01%	0.071	0.000	1.38%	0.133
		Stat-Pak : LST	0.010	7.32%	0.144	-0.00001	0.03%	0.139
		LST : FPA	0.005	4.23%	0.334	0.011	34.70%	0.249
	Classical Two-Stage	Stat-Pak : FPA	0.139	100.00%	1.000	0.023	22.51%	0.162
		Stat-Pak : LST	0.000	0.00%	0.000	0.014	17.77%	0.178
		LST : FPA	0.000	0.00%	0.000	-0.014	19.74%	0.130

Table 3.6 Predictive values, post-test probability of infection, and diagnostic odds ratios for each test and pairwise and three-parallel test combinations of each test for diagnosis of *M. bovis* in elk from southern Manitoba using classical two-stage, Bayesian and validation subsample approaches (5.57% prevalence assumed).

Validation Approach	Test Combination	Predictive Value Positive[c]	Predictive Value Negative[c]	Likelihood Ratio Positive	Likelihood Ratio Negative	Post-test probability Positive[c]	Post-test probability negative[c]	Diagnostic Odds Ratio[d]	AUC[e]
Classical Two Stage (uncorrected)	FPA	21.50%	96.50%	4.63	0.62	0.2126	0.0347	7.49	0.673
	Stat-Pak	48.20%	96.50%	15.75	0.39	0.4819	0.0225	40.34	0.793
	LST	29.00%	99.20%	6.93	0.14	0.2908	0.0079	20.65	0.877
	FPA + Stat-Pak[b]	25.90%	98.30%	5.91	0.29	0.2593	0.0167	20.65	0.812
	FPA + LST[b]	21.70%	99.70%	4.71	0.05	0.2188	0.0030	93.76	0.878
	LST + Stat-Pak[b]	27%	99.70%	6.2	0.05	0.2701	0.0030	125.91	0.902
	FPA + Stat-Pak + LST[b]	20.90%	100%	4.4	0.02	0.2063	0.0000	177.68	0.889
Classical Two-Stage (bias corrected)	FPA	12.82%	95.14%	2.49	0.87	0.1389	0.0483	2.88	0.561
	Stat-Pak	29.32%	96.19%	7.03	0.67	0.3918	0.0374	10.47	0.656
	LST	19.10%	97.38%	4.00	0.46	0.2229	0.0254	8.78	0.730
	FPA + Stat-Pak[b]	18.81%	96.70%	3.93	0.58	0.2188	0.0322	6.79	0.684
	FPA + LST[b]	15.96%	97.81%	3.22	0.38	0.1794	0.0212	8.48	0.742
	LST + Stat-Pak[b]	21.18%	99.09%	4.55	0.16	0.2537	0.0087	29.13	0.841
	FPA + Stat-Pak + LST[b]	19.19%	100.00%	4.03	0.00	0.2243	0.0000	ND	0.876
Bayesian (CID model)	FPA	26.40%	96.30%	6.08	0.66	0.2642	0.0374	9.22	0.658
	Stat-Pak	44.90%	98.50%	13.8	0.25	0.4493	0.0146	55.3	0.851
	LST	16.50%	97.90%	3.36	0.369	0.1653	0.0212	9.11	0.749
	FPA + Stat-Pak[b]	30.50%	99%	7.45	0.164	0.3051	0.0096	45.3	0.870

	FPA + LST[b]	15.60%	98.60%	3.14	0.246	0.1561	0.0143	12.8	0.779
	LST + Stat-Pak[b]	17.70%	99.40%	3.65	0.0975	0.1770	0.0057	37.4	0.837
	FPA + Stat-Pak + LST[b]	15.90%	99.70%	3.2	0.052	0.1590	0.0031	61.6	0.832
Validation Subsample	FPA	66.20%	95.50%	33.2	0.805	0.6622	0.0453	41.3	0.597
	Stat-Pak	25.70%	97.40%	5.85	0.446	0.2565	0.0256	13.1	0.749
	LST	11.50%	96.90%	2.21	0.549	0.1150	0.0314	4.02	0.664
	FPA + Stat-Pak[b]	30.10%	98.70%	7.29	0.225	0.3007	0.0131	32.5	0.845
	FPA + LST[b]	11.30%	96.80%	2.16	0.554	0.1127	0.0317	3.9	0.661
	LST + Stat-Pak[b]	13.70%	100%	2.47	0.132	0.1372	0.0000	18.7	0.819
	FPA + Stat-Pak + LST[b]	13.50%	100%	2.43	0.134	0.1357	0.0000	18.1	0.736

[a] – Assumes that all serially negative elk (negative on all 3 tests) were true negatives (based on 100% NPV from validation subsample).
[b] – Assumes sensitivity covariance and specificity covariance between FPA and Cervid TB Stat-Pak and conditional independence between other LST and other two tests.
[c] – Assumes conditional independence between all three tests
[d]- Elk that were validated by necropsy & culture without prior blood testing (Capture mortalities & culled animals). Assumes a pre-test probability of infection (prevalence) of 5.57% (Bayesian estimate from conditional independence model)
[e] – Ratio of likelihood ratio positive to likelihood ratio negative.
[f] – Area under the curve from ROC curve.

Table 3.7 Sensitivity analysis for Bayesian latent class model using partially informative prior distributions for the elk conditional dependence (CD) Bayesian model.

		Informative Prior Distribution	Uniform Prior Distribution [a]	Difference (%)
Sensitivity	FPA	0.3699	0.2347	36.6
	Stat-Pak	0.7636	0.3689	51.7
	LST	0.7144	0.6922	3.1
Specificity	FPA	0.923	0.944	2.1
	Stat-Pak	0.945	0.929	2.1
	LST	0.787	0.846	3.4

[a] - Using flat uniform priors for Se (a=0.2, b=0.8) and for Sp (a=0.5, b=1) for all three tests and beta (1,1) for prevalence of both WCZ and Outside of WCZ.

Table 3.8 Sensitivity, specificity and predictive values of white-tailed deer tested for *M. bovis* with FPA, LST, Stat-Pak and combinations from southern Manitoba using a classical two-stage approach.

	Se (95% CI)		n	Sp (95% CI)		n	PPV (95% CI)		NPV (95% CI)	
Stat-Pak	0.33	0.008 , 0.91	3	0.963	0.929 , 0.984	217	8.4%	2.3 , 28.8	99.3%	98.6 , 99.7
FPA	0.33	0.008 , 0.91	3	0.926	0.883 , 0.957	217	4.4%	1.3 , 16.1	99.3%	98.6 , 99.7
LST	1	0.29 , 1	3	0.962	0.926 , 0.983	210	21.0%	9.3 , 31.8	100.0%	98.3 , 100
Stat-Pak/LST	1	0.29 , 1	3	0.933	0.891 , 0.963	210	13.2%	6.5 , 19.3	100.0%	98.2 , 100
Stat-Pak/FPA	0.33	0.008 , 0.91	3	0.889	0.839 , 0.927	216	2.9%	0.9 , 11.1	99.2%	98.5 , 99.7
FPA/LST	1	0.29 , 1	3	0.886	0.835 , 0.926	211	8.2%	4.3 , 11.5	100.0%	98.1 , 100
All three tests	1	0.29 , 1	3	0.858	0.803 , 0.902	211	6.6%	3.6 , 9.2	100.0%	98.1 , 100

Table 3.9 Likelihood ratios, post-test probabilities, diagnostic odds ratio and AUC of white-tailed deer tested for *M. bovis* with FPA, LST, Stat-Pak and combinations from southern Manitoba using a classical two-stage approach.

	Post Test Pr. Positive	95% CI	Post Test Pr. Negative	95% CI	DOR	95% CI	AUC	95% CI
Stat-Pak	0.08	0.02 , 0.29	0.007	0.003, 0.014	14.79	1.74 , 125.3	0.65	0.32 , 0.98
FPA	0.05	0.01 , 0.16	0.007	0.003, 0.015	7.33	0.91 , 59.0	0.63	0.3 , 0.96
LST	0.21	0.1 , 0.32	0	0 , 0.018	166.76	7.96 , 3492	0.98	0.97 , 0.99
Stat-Pak/LST	0.13	0.07 , 0.19	0	0 , 0.018	94.86	4.67 , 1926	0.97	0.95 , 0.98
Stat-Pak/FPA	0.03	0.01 , 0.12	0.008	0.003 ,0.015	4.71	0.6 , 37.3	0.61	0.28 , 0.94
FPA/LST	0.08	0.05 , 0.12	0	0 , 0.019	53.57	2.69 , 1068	0.94	0.92 , 0.96
All three tests	0.07	0.04 , 0.09	0	0 , 0.02	41.66	2.1 , 827	0.93	0.91 , 0.95

Table 3.10 Sensitivity, specificity and predictive values of white-tailed deer (n = 80) tested for *M. bovis* with FPA, LST, Stat-Pak and combinations from southern Manitoba using a validation subsample.

	Se	95% CI	n	Sp	95% CI	n	PPV	95% CI	NPV	95% CI
Stat-Pak	1	0.025 ,1	4	0.962	0.89 , 0.99	75	20.8%	4.5 ,38.5	100.0%	97.2 ,100
FPA	1	0.025 ,1	3	0.974	0.91 , 1	76	28.3%	5.3 ,50.7	100.0%	97.2 ,100
LST	1	0.025 ,1	6	0.931	0.85 , 0.98	67	12.7%	3.1 ,23.8	100.0%	97.1 ,100
Stat-Pak/FPA	1	0.025 ,1	6	0.935	0.86 , 0.98	72	13.5%	3.3 ,25.1	100.0%	97.1 ,100
Stat-Pak/LST	1	0.025 ,1	9	0.889	0.79 , 0.95	64	8.3%	2.3 ,15.3	100.0%	96.9 ,100
FPA/LST	1	0.025 ,1	8	0.903	0.81 , 0.96	65	9.4%	2.5 ,17.4	100.0%	97 ,100
All three tests	1	0.025 ,1	11	0.861	0.76 , 0.93	62	6.8%	1.9 ,12.3	100.0%	96.8 ,100

Table 3.11 Likelihood ratios, post-test probabilities, and diagnostic odds ratio of white-tailed deer (n = 80) tested for *M. bovis* with FPA, LST, Stat-Pak and combinations from southern Manitoba using a validation subsample.

Test	LR+	95% CI	LR-	95% CI	Post Test Pr Pos	95% CI	Post Test Pr Neg	95% CI	DOR	95% CI
Stat-Pak	16.93	4.6 ,62.1	0.26	0.02 ,2.89	0.79	0.61 ,0.95	1.0	0.97 ,1.0	64.71	2.2 ,1892
FPA	23.7	5.5 ,62.1	0.26	0.02 ,2.85	0.72	0.49 ,0.94	1.0	0.97 ,1.0	91.8	2.9 ,2862
LST	9.95	3.2 ,62.1	0.27	0.02 ,2.98	0.87	0.76 ,0.97	1.0	0.97 ,1.0	36.82	1.3 ,1015
Stat-Pak/FPA	10.64	3.4 ,62.1	0.27	0.02 ,2.97	0.86	0.75 ,0.97	1.0	0.97 ,1.0	39.55	1.4 ,1089
Stat-Pak/LST	6.44	2.3 ,62.1	0.28	0.03 ,3.12	0.92	0.85 ,0.98	1.0	0.97 ,1.0	22.76	0.9 ,605
FPA/LST	7.3	2.6 ,62.1	0.28	0.03 ,3.08	0.91	0.83 ,0.97	1.0	0.97 ,1.0	26.2	1 ,702
All 3	5.21	2 ,62.1	0.29	0.03 ,3.23	0.93	0.88 ,0.98	1.0	0.97 ,1.0	17.86	0.7 ,468

Table 3.12 Sensitivity, specificity, prevalence and covariance values for white-tailed deer tested for *M. bovis* with FPA, LST, Stat-Pak and combinations from southern Manitoba using a Bayesian latent class analysis.

Test	Sensitivity	95% CI[1]	Specificity	95% CI	PPV	95% CI	NPV	95% CI
Stat-Pak	0.516	0.27 , 0.76	0.98	0.97 , 0.99	0.231	0.095 , 0.47	0.994	0.991 , 1
FPA	0.558	0.24 , 0.85	0.912	0.87 , 0.94	0.069	0.021 , 0.14	0.994	0.99 , 1
LST	0.746	0.6 , 0.86	0.857	0.82 , 0.89	0.057	0.037 , 0.08	0.997	0.994 , 1
Stat-Pak/LST	0.877	0.797 , 1	0.84	0.795 , 0.88	0.06	0.042 , 0.09	0.998	0.997 , 1
Stat-Pak/FPA	0.877	0.536 , 1	0.894	0.844 , 0.93	0.088	0.037 , 0.14	0.998	0.994 , 1
FPA/LST	0.888	0.696 , 1	0.782	0.713 , 0.84	0.045	0.027 , 0.07	0.998	0.995 , 1
All three tests	0.923	0.832 , 1	0.766	0.692 , 0.83	0.044	0.03 , 0.06	0.999	0.997 , 1
Secov[2]	-0.0906	-0.372 , 0.154						
Spcov[3]	0.0004	-0.002 , 0.007						
WCZ prevalence	1.153%	0.33% , 2.78%						
Outside prevalence	0.122%	0.01% , 0.45%						

[1] 95% credible interval
[2] Sensitivity covariance for conditional dependence model (assumes dependence between FPA & Stat-Pak)
[3]Specificity covariance from conditional dependence model (assumes dependence between FPA & Stat-Pak)

Table 3.13 Likelihood ratios, diagnostic odds ratio (DOR), and AUC values for white-tailed deer tested for *M. bovis* with FPA, LST, Stat-Pak and combinations from southern Manitoba using a Bayesian latent class analysis.

Test	LR+	95% CI	LR-	95% CI	DOR	AUC[1]	95% CI
Stat-Pak	25.8	9.0 , 76.0	0.494	0.24 , 0.75	52.2	0.748	0.62 , 0.88
FPA	6.34	1.8 , 14.2	0.485	0.16 , 0.87	13.1	0.735	0.555 , 0.9
LST	5.22	3.3 , 7.8	0.296	0.16 , 0.49	17.6	0.802	0.71 , 0.88
Stat-Pak/LST	5.48	3.8 , 8.3	0.146	0 , 0.27	37.5	0.859	0.79 , 0.94
Stat-Pak/FPA	8.27	3.3 , 14.3	0.138	0 , 0.56	59.9	0.886	0.685 , 0.97
FPA/LST	4.07	2.4 , 6.3	0.143	0 , 0.44	28.5	0.835	0.7 , 0.92
All three tests	3.94	2.7 , 5.9	0.101	0 , 0.25	39	0.845	0.76 , 0.92

[1] Area under the curve, calculated as Se + Sp

Table 3.14 Parameters for final multivariate logistic regression using parallel interpretation of three blood-based assays as outcome variable[1]

Variable	Factor Level	Odds Ratio	Std. Err.	z	P > z	95% CI	
Year		0.916	0.026	-3.05	0.002	0.866	0.969
Sex	Female	ref	ref	ref	ref	ref	ref
	Male	0.664	0.096	-2.83	0.005	0.500	0.881
Park	Outside RMNP	ref	ref	ref	ref	ref	ref
	Inside RMNP	0.571	0.152	-2.1	0.036	0.339	0.963
Species	WTD	ref	ref	ref	ref	ref	ref
	Elk	2.379	0.464	4.44	0	1.623	3.487
intercept		0.731	0.201	-1.14	0.254	0.427	1.253

[1] – Data for regression restricted to sampling year 2004/2005 and later and for animals with all 3 tests only

CHAPTER 4: RISK FACTORS FOR INFECTION IN WILD CERVIDS IN THE GREATER RIDING MOUNTAIN ECOSYSTEM

The research undertaken in Chapter 4 seeks to understand the critical risk factors important in the maintenance of M. bovis infection in the GRME by looking at a larger set of risk factors in a multivariate framework. The risk factors which were identified in Chapter 2 were examined individually and did not include co-infection with other agents/parasites or examine interactions between factors, or examine important risk factors such as elk density and density of barrier fencing. Understanding these factors and their relative importance is critical for; 1) providing risk estimates for the disease freedom model developed in Chapter 6, 2) providing refined estimates for proving disease freedom in cattle in RMEA, and 3) creating an understanding of why transmission among wild cervids apparently ceased in this episystem and which management factors were likely responsible.

4.1 Introduction

Wildlife reservoirs of *M. bovis* have been identified as emergent diseases worldwide causing significant spillover to domestic animal populations and extreme difficulty in controlling spread of infection (Michel et al., 2006; Fitzgerald and Kaneene, 2013) once spillover occurs into wild populations. Wild ungulate species including red deer, African buffalo, fallow deer, white-tailed deer, wild boar and elk have been implicated as reservoir species either separately or in combination, and yet some of these species have been designated as spillover hosts or competent reservoir hosts depending on environmental and demographic characteristics (Naranjo et al., 2008; Palmer et al., 2012; Palmer, 2013). This has led some authors to characterize wild cervids and others as density-dependent reservoir hosts (Hunter, 1996; Lugton et al., 1998; Vicente et al., 2007a), that maintain *M. bovis* independently, but only when at high densities, or along with other environmental factors which force wild cervids into close proximity (e.g. artificial feeding, waterholes). In other parts of the world where wild cervids have been implicated as reservoir hosts for *M. bovis*, risk factors such as age, sex, animal density, environmental factors, and presence of other reservoir hosts have been implicated as risk factors for maintenance of infection (Palmer, 2013).

This study builds on previously published research that conducted a preliminary exploratory analysis of potential risk factors using contingency table analysis (Shury and Bergeson, 2011). This study uses a larger dataset spanning 1997 to 2013 and utilizes advanced model building techniques to explore confounding, interaction and multilevel characteristics of this dataset as well as a larger suite of explanatory variables. Formal surveillance for *M. bovis* in the GRME began in 1997, although the first positive elk was found in 1992. The objective of this research is to develop an explanatory model which explains the relationship between the outcome variable (culture positivity at necropsy) and a set of explanatory variables for elk and white-tailed deer in the GRME. Model results will be interpreted to understand why and how *M. bovis* has been maintained in the GRME and to further understand the host status of wild cervids in relation to *M. bovis* infection dynamics.

4.2 Methods & Materials

Data were collected from long-term structured surveillance for in wild cervids from the area in and around Riding Mountain National Park (RMNP) conducted jointly by Manitoba Conservation and the Parks Canada Agency (Shury and Bergeson, 2011; Shury et al., 2014). This consisted of samples collected from sport hunters through licensed hunting activities (hunt sample), samples collected from wildlife found dead which included primarily road killed animals and predator killed animals (opportunistic sample), animals culled for density reduction programs (culled sample), and animals that were blood tested with removal of positives or suspects (blood test sample). White-tailed deer and elk were the only two species included in the database; moose were excluded as no *M. bovis* positive moose have been found since surveillance began, despite having sampled approximately 630 moose between 1997 and 2013. Hunter sampled animals were collected through an active communications program that required hunters to provide a complete head and lung pluck from harvested animals collected through the

months of August to February every year of the program from 1997 to 2013. A complete set of head and lungs was not always obtained and many samples were either too autolyzed or damaged by gunshot trauma to be useful. Opportunistic samples were collected when found through public reporting or through other surveillance programs (radio-telemetry). Non-selective culling occurred sporadically throughout the study period with an area-wide surveillance cull of 226 white-tailed deer in March of 2004 through ground-based agency sharpshooting in the entire Riding Mountain Eradication Area (RMEA). Selective culling for both elk and white-tailed deer occurred within a designated Core Area (Figure 4.1) during the winters of 2008/2009, 2009/2010 and 2013/2014 with the primary goal of reducing elk density, as there was evidence that cervid density contributes to transmission and maintenance of *M. bovis* in cervid populations (O'Brien et al., 2006; Vicente et al., 2007a). Both selective and non-selective culling of white-tailed deer was primarily done to both reduce density and determine where infected deer were present, as most samples were obtained through hunting outside Riding Mountain National Park, and very few samples were obtained through opportunistic sampling.

Blood testing for *M. bovis* in elk began in the winter of 2000/2001and was continued annually until 2013/2014, while blood testing for white-tailed began in the winter of 2004/2005 continuing annually until 2013/14. Animals were captured using helicopter net gunning during winter and late spring (December through May) using methods described in detail in Shury et al. (2014). Briefly, adult cow elk were initially captured in January or February, while adult bull elk were captured in April after antlers were dropped. A two-stage sampling strategy was employed for the blood testing surveillance component. Three different blood based assays were used as initial screening tests interpreted in parallel; a fluorescent polarization assay (FPA), a lymphocyte stimulation test (LST) and a lateral flow chromatographic assay (Cervid TB Stat-

Pak). Animals that were positive on any one of these three tests were recaptured 1 to 60 days later, euthanized with a captive bolt gun, and subjected to a complete necropsy examination and culture for *Mycobacterium bovis*. Animals that were negative on all three screening tests were not recaptured unless by chance in subsequent capture years through the blood testing program.

Age of wild cervids was initially estimated based on tooth wear to one of five age categories; <1 year, 1 to 3 years, 4 to 5 years, 6 to 8 years, and >= 9 years. Animals that were culled, euthanized because of a positive blood test, or hunter killed animals that had gross visible lesions compatible with *M. bovis* were more precisely aged by examination of cementum annuli from incisor teeth collected at necropsy (Matson's Lab LLC, Missoula, MT). Age category of animals that were euthanized were back-corrected using the cementum age at necropsy rather than the initial estimate. The final database consisted of 14,466 records of individual elk and white-tailed deer. After 230 (1.6%) records were removed from the database because of poor sample quality or other issues, leaving a total of 14,236 records used for the analysis.

An initial logistic regression model was developed using *M. bovis* culture status (positive or negative) as the outcome variable and a set of 13 potential risk factors as predictor variables using STATA IC 13.0 for Windows (STATACorp LP, College Station, TX). The inclusion of elk density restricted the number of observations substantially (reduced by ~50%), so an alternate unrestricted model was also explored which did not include elk density as a predictor variable. Elk density was estimated from annual aerial 25% and 100% coverage surveillance flights conducted within RMNP and extending outside the park boundary for one to 5 kilometers (Vander Wal et al., 2013b) and was specified to the level of subzone (Figure 1). A variable describing the density of barrier fencing (fences per square kilometre) was calculated annually for each subzone. This was calculated as the cumulative number of barrier fences erected per

year in each subzone divided by the total area of the subzone in square kilometres. Year of sampling was categorized as a factor variable with four categories which reflected the different management changes that occurred on the landscape over the period of study. A pre-management phase (1997/98 to 2000/2001), an intensive management phase when the majority of hay barrier fencing (~80%) to protect stored hay in the RMEA were installed and elk densities were initially reduced (2001/02 to 2004/05), an interim management phase (2005/06 to 2009/10), and a post-management monitoring phase (2010/11 to 2013/14) when barrier fencing was essentially complete, but elk and deer management in the core area continued. All variables were initially screened using univariate logistic regression and variables with a p-value <= 0.2 were retained in the model. This model was further refined using stepwise backward selection until all predictor variables remaining were significant ($p < 0.5$). Species (elk or white-tailed deer) was the only predictor forced into the model regardless of statistical significance due to its biological importance, its potential for confounding and a desire to obtain final estimates that allowed stratification by this variable. Only animals that were killed and had valid post-mortem culture results were used in the analysis. Animals released as a result of being blood-test negative were excluded, even though these test negative animals had a negative predictive value of 100% (Shury et al., 2014). All two-way interactions between final significant dependent variables were assessed by including an interaction term in the model. Interaction terms with $p < 0.05$ were excluded from the final model. Variables excluded from the model due to statistical significance were added back in to check for confounding, and any variables that changed parameter estimates of final main effects variables by more than 20% were retained in the model. Model fit was assessed through examination of Hosmer-Lemshow test statistic and examination of the predicted ROC (receiver operating characteristic) curve and area under the curve (AUC). Trends

in prevalence over time for elk and white-tailed deer separately and combined were evaluated using the ptrend package in Stata 13.1 (Royston 2002). Results from the final model were transformed into the probability scale using the predict command in STATA 13.1, to allow visualization of predicted probabilities.

4.3 Results

Information on 14,226 wild cervids, which comprised 9,562 white-tailed deer (67.2%) and 4,674 elk (32.8%), was collected through the four surveillance streams utilized for this study (Table 4.1). The majority of samples came from hunter harvested animals for both species, and a higher proportion of white-tailed deer were sampled through culling than elk (Table 4.2). Significantly more elk were sampled through blood sampling than deer (chi square =724.0, $p<0.0001$), as elk have been considered the primary reservoir *of M. bovis* in this ecosystem (Lees et al., 2003; Shury and Bergeson, 2011).

4.3.1 Restricted Logistic Regression Model

Due to limitations on where elk density estimates were available within the RMEA (n = 8,025), a restricted logistic regression model was used to explore the relationship of a set of risk factors that included elk density. Model fit parameters for the restricted model indicated adequate fit using Hosmer-Lemeshow ($\chi 2 = 6.28$, $p = 0.616$) goodness of fit statistics. The Hosmer-Lemeshow GOF statistic using 10 quantile categories is likely a better indicator of fit due to the large number of covariate patterns in this model (1328). The area under the curve for the restricted model indicates an excellent model fit (AUC = 0.933, Figure 4.2). Six variables were significant in the restricted model (Table 4.3); year category, age category, sex, surveillance method, whether animals were in the core area, and elk density. Species was forced into the model as a seventh dependent variable, due to its biological significance. Density of barrier

fencing was not a significant variable in the final model. No significant interactions were found and no variables that were excluded due to statistical significance were considered confounders.

Sampling phase (year category) exhibited a strongly decreasing odds ratio compared to the pre-management phase (reference category) in the restricted model, indicating that overall, the odds of finding culture positive cervids through surveillance decreased over time (Table 4.3). The odds of finding a culture positive cervid through surveillance in the intensive management phase (2001 to 2005) decreased 53% on average compared to the pre-management phase (1997 to 2001). This decrease continued in the interim management phase (2006 to 2010) with the odds of finding a culture positive cervid decreasing 68.9% on average and by the post-management phase (2011 to 2014), the odds had decreased 94.0% on average compared to the pre-management phase. This trend is graphically apparent in Figure 4. 4 when visualized on the probability scale and also demonstrates the strong relationship between elk density and the probability of being culture positive.

The odds of being culture positive increased with increasing age category (Table 4.3) as well. This increase was roughly linear on the probability scale for both species and sexes, with the exception of white-tailed deer (Figure 4.5), which exhibited a sharp increase in probability for both male and female deer greater than 8 years of age. Animals sampled in the Core area had 15.7 times higher odds of being culture positive than animals sampled outside this area (Table 4.3) with probabilities that differed markedly and decreased over time in both zones (Figure 4.6). Male animals had 2.06 times higher odds of being culture positive than female animals with probabilities that varied by age and sex (Figure 4.5). Both male and female white-tailed deer exhibited a sharp increase in probability of being culture positive for the oldest age category. Compared to hunted animals, opportunistic, culled and blood sampled animals had higher odds

of being culture positive. Probabilities of being culture positive showed a corresponding linear increase for both species for each of these surveillance components (Figure 4.8). Mean probability of being culture positive exhibited an increase during the intensive management phase (2001 to 2005), which has subsequently decreased for elk, while remaining very low throughout the period of surveillance compared to elk, but still declining over time (Figure 4.9). The odds of being culture positive increased 3.4 times on average, for every one square kilometer increase in elk density. Mean elk density was consistently higher within RMNP compared to outside the park, except in the year category (post management phase, 2011 to 2014), when elk densities become similar inside and outside RMNP (Figure 4.6). Elk density fluctuated significantly between 2000 and 2013, but maintained consistent patterns of density concentration over this time period with higher densities consistently occurring west of Highway 10 within RMNP (Figure 4.7). Since 1976, elk densities within RMNP have been consistently above one elk per square kilometer except for a brief period in the mid 1980's and exhibited a sharp decline beginning in 2000 (Figure 4.15). This latter decline in the early 2000's coincides temporally with a sharp increase in the number of farms with fenced hay storage yards which were erected to protect stored hay from elk in winter (Figure 4.14).

4.4 Unrestricted Logistic Regression Model

The unrestricted model (Table 4.4) had the same significant risk factors in the final model and provided somewhat different estimates for key risk factors, not including elk density but used a much larger sample of animals (n= 14,060). Density of barrier fencing was also not a significant risk factor in the unrestricted model and no interaction or confounding was discovered. Model fit parameters for this model were also very similar, with a non-significant Hosmer-Lemeshow test using 10 quantile categories ($\chi 2 = 7.26$, $p = 0.508$), and an AUC of 0.955. The number of covariate patterns in the unrestricted model was much reduced at 286, due to the exclusion of elk

density as a risk factor in the final model. Model fit appears to be adequate for both the restricted and unrestricted models. Similar to the restricted model, mean probability of being culture positive has decreased (Figure 4.10) since 1997. The estimates of this decrease in mean probability over time were larger than the restricted model. Elk density proved to be a confounder in the final restricted model, changing parameter estimates of sampling category, core area, species and capture method by > 30% compared to the unrestricted model. For this reason, the restricted model is considered to be a better model for estimation of parameter estimates as it includes elk density, a confounding variable.

4.5 Prevalence and Age changes over time

The mean age of *M. bovis* positive elk exhibited an increasing trend over the period of study, especially in the post-2004 sample (Fig. 4.11). There was no significant change in prevalence for elk or white-tailed deer separately (Figure 4.12) over the time course of the study (1997 to 2014, elk: $\chi^2 = 0.26$, p=0.610, WTD: $\chi^2 = 1.88$, p=0.170), but when both species were combined there was a significant decline over the entire time course ($\chi^2 = 8.93$, p = 0.0028). There was also a significant trend for elk alone between 2003 and 2014 ($\chi^2 = 5.55$, p =0.018), but this trend was marginally non-significant for white-tailed deer ($\chi^2 = 3.39$, p = 0.065), but was highly significant for both species combined ($\chi^2 = 14.80$, p =0.0001). Period prevalence was always higher for elk than WTD and was highest during the intensive and interim management phases, than either the pre-management or post-management phase for both species (Figure 4.13).

4.6 Discussion

Different risk factors have been identified for wildlife reservoirs of *M. bovis*, but these are often dependent on local and regional circumstances which vary widely from one geographic location to another (Lugton et al., 1997; Lugton et al., 1998; O'Brien et al., 2002; Nugent, 2005; Vicente et al., 2007b; Gortazar et al., 2008; Palmer, 2013). In the state of Michigan where *M.*

bovis has been maintained by free-ranging white-tailed deer for several decades, baiting, feeding and deer density have been identified as significant factors which are maintaining this wildlife reservoir (O'Brien et al., 2011a). In Spain, the presence of multiple reservoir species and local congregation at water holes allows maintenance of wildlife reservoirs involving wild boar, red deer and cattle (Gortazar et al., 2008; Naranjo et al., 2008; Romero et al., 2008; Martin-Hernando et al., 2010), while in New Zealand wild red deer require contact with high density brush-tailed possum populations to maintain infection and are considered spillover hosts in the absence of this condition (Lugton et al., 1998; Nugent, 2005; Ryan et al., 2006a).

In this study, six significant factors (from the restricted model) were found to be associated with *M. bovis* culture positive results in two wild species of cervid. A previous study showed prevalence of *M. bovis* culture positive cervids declining significantly since 2003 when a suite of management measures were initiated including barrier fencing to protect hay bales in winter, extended hunting seasons, density reduction through culling, legislated bans on baiting and feeding, prescribed burning to improve elk habitat, and selective removal using three blood tests in parallel (Shury and Bergeson, 2011). Since that time, prevalence has continued to decline and there have now been two concurrent years (2011/12 and 2012/13) of intensive surveillance in elk and deer populations (zero out of 1,761 cervids tested), which has not found any culture positive animals (Figure 4.10). One of the major reasons for this decline in prevalence can be attributed to decreasing elk density since 2003, in combination with other management activities undertaken at that time.

This is one of the few empirical studies that has demonstrated a strong positive relationship between reduced density and prevalence of *M. bovis* in a wildlife reservoir. Elk density reduction (Figures 4.7 & 4.14) was achieved both through extended hunting seasons for elk and through

active agency-sponsored non-selective culling which was carried out through helicopter net gunning within RMNP and euthanasia of elk and deer by captive bolt gun. It is likely that elk density was not the only factor involved in the rapid reduction in prevalence seen in this episystem; reduced contact with infected cattle around RMNP was likely another key factor (Brook et al., 2013; van Beest et al., 2013; Vander Wal et al., 2013a; Vander Wal et al., 2013b; Gooding and Brook, 2014). Elk density was in decline prior to 2003 (Figure 4.12) at roughly the same time that hay barrier fencing began in earnest. Although density of barrier fencing was not a significant risk factor in the final logistic regression model, there is evidence to support the role of this management factor as being important in the resulting prevalence reduction. Damage claims by ranchers in the RMEA have declined dramatically since these fences were first constructed (Gooding and Brook, 2014) and farmer observations and telemetry data indicate that elk visitation to these sites has decreased markedly (Brook, 2010).

Historically, elk density was relatively high within RMNP (Fig. 4.14), and this is likely one reason why *M. bovis* has persisted in this landscape for over 35 years, based on two wolves that were found to be infected with the same spoligotypes of *M. bovis* present currently (Lutze-Wallace et al., 2005a). Elk density reduction was only carried out after careful consideration and consultation with major stakeholders, some of whom did not agree with this course of action as a management strategy (Brook and McLachlan, 2006).

The Scientific Review Committee for the Manitoba TB Task Force carried out population modeling using both stochastic and deterministic models demonstrating that elk density reduction would still allow the elk population to recover to prior levels within approximately 5 to 10 years (pers. comm. P. Paquet 2013, SRC chair). This convinced government agencies and

most of the impacted stakeholders that it would be a worthwhile disease management strategy to pursue.

Non-selective culling of wildlife populations to manage disease, especially *M. bovis*, has been extremely controversial in other countries and geographic regions (O'Connor et al., 2012; Anonymous, 2013a; Munro, 2013; Woolhouse and Wood, 2013). In Michigan, surveys of hunters, who are major stakeholders in funding and managing disease in that state, have demonstrated that agency-sponsored non-selective culling will be strongly opposed and not accepted as a management option (O'Brien et al., 2011a). In contrast, agency sponsored non-selective culling of white-tailed deer in Minnesota played a major role in deer density reduction and removal of a potential wildlife reservoir in that state (Carstensen et al., 2011).

Selective culling of elk and white-tailed deer using blood tests as a screening tool and only removing blood test positive animals was generally widely accepted as a management tool in the GRME, compared to non-selective culling, as fewer elk were removed. This was only possible because relatively sensitive and specific tests that targeted both humoral and cell-mediated immunity were able to screen elk for removal, albeit with a relatively large proportion of false positives (Shury et al., 2014). This substantially increased the overall cost of disease management in the GRME, but struck a compromise between public and stakeholder tolerance and disease management objectives; a fine balance that can be difficult to achieve when dealing with a wildlife reservoir (Palmer, 2013). One only has to look at the controversy over badger culling in the United Kingdom to understand how difficult this can be when a highly-valued wildlife species is the demonstrated *M. bovis* reservoir (Anonymous, 2013a; 20132013b; Woolhouse and Wood, 2013).

This study provides further evidence of the primary reservoir status of elk and the spillover status of white-tailed deer at current densities in this episystem. Prevalence in white-tailed deer was consistently lower than prevalence in elk throughout the entire study period, despite the majority of tested animals being white-tailed deer (66.4%). In this analysis, we found lower probabilities of being culture positive for white-tailed deer compared to elk in all sampling categories across years. Unless baiting and feeding of wildlife resumes and/or white-tailed deer densities are allowed to increase to levels approaching those in the state of Michigan, it is very unlikely that white-tailed deer could be considered reservoir hosts in the context of the GRME. Hosts such as white-tailed deer, wild boar and ferrets which can be either spillover hosts or reservoir hosts for *M. bovis* depending on local environmental circumstances, should be referred to as facultative hosts, because the environment in which they exist (i.e local and regional density) determines their reservoir status.

In general, this study and others have demonstrated that cervids are not consistently ideal reservoir hosts for *M. bovis*. In Michigan elk are considered a spillover host due to geographical separation from the core area of infection and low densities compared to white-tailed deer (O'Brien et al., 2008). Case reports of sporadic *M. bovis* infection in wild cervids from throughout North America during the eradication of bovine tuberculosis from domestic cattle herds in the 20th century (Friend et al., 1963; Rhyan et al., 1995; Wobeser, 2009) indicate that spillover to wild cervids was likely a relatively common event, as under reporting of cases was very likely at this time in general. The fact that these cases did not result in a persistent wildlife reservoir of *M. bovis*, with the possible exception of Michigan and Manitoba, suggests that wild cervid densities were likely below the critical community threshold required for disease establishment with density-dependent diseases such as *M. bovis*.

Density of white-tailed deer in Michigan has recently ranged between 16-18 deer/km^2, while in Minnesota deer densities are an order of magnitude lower (between 1 and 3 deer/km^2)(Carstensen et al., 2011), similar to that observed in southern Manitoba. Hickling (2002) and Obrien et al (2011a) have suggested that a critical community threshold density occurs at approximately 10-12 deer/km^2.

This study supports the principle of a potential threshold in wild cervid populations and suggests that the required density may be even lower, depending on extent of local baiting and feeding. Based on the inability of *M. bovis* to establish in areas outside the core area in the western GRME, despite having been present since at least the late 1970's, a potential threshold for establishment in elk seems to occur around 1 elk/km^2. Once elk densities in the core area declined to below this level, prevalence declined and for the past 3 years, no *M. bovis* positive elk have been detected, despite relatively intensive surveillance. Another critical factor which limited the spread of *M. bovis* from the relatively small spatial area of the core area (1366 sq km), was the strong genetic structure in the elk population in this area (Vander Wal et al., 2012a). This likely prevented long-distance translocation of the disease, despite the lack of population structure in white-tailed deer populations (Vander Wal et al., 2013b).

Several limitations are present within this study that bear mentioning. First, elk density was very crudely estimated based on aerial surveys, which are error prone due to limitations imposed by sightability, habitat type, weather, snow conditions, and observer bias (Noyes et al., 2000). Important ecological correlates such as habitat and predation were not examined in this modelling framework, which have previously been shown to affect elk group size and density (Hebblewhite and Merrill, 2007; Vander Wall, 2011; Vander Wal et al., 2013a; Vander Wal et al., 2013b; van Beest et al., 2014a; van Beest et al., 2014b). While this was a crude estimate,

other studies have used similar crude estimates to successfully examine risk factors for brucellosis at a broad scale (Cross et al., 2010). Culture from tissues at necropsy was used as the outcome for this study, but culture is known to have relatively low sensitivity, especially for early and latent infections (Murphy et al., 2010; Corner et al., 2011). Although this is considered the gold standard for *M. bovis* infections in wildlife (de Lisle et al., 2005), some infected animals were likely missed, underestimating the effect in some of the important risk factors. This study was essentially an extended, cross-sectional study that examined both outcome and risk factors at the same time. While baiting and feeding was also likely an important risk factor for culture positivity in wild cervids, it was a very difficult parameter to measure precisely and so was not included in this model. Despite these limitations, this is still the first comprehensive epidemiological evaluation of important risk factors in this ecosystem, and will inform and guide future management in this and other wildlife reservoirs of disease.

In summary, six significant risk factors, including elk density, were found to be significantly associated with *M. bovis* culture positive results in the GRME episystem. The factors most strongly associated with culture positivity were geographical location (within core area), elk density and year category when animals were sampled. Similar to other studies in wild cervids, age and sex were also significant risk factors for culture positivity in this system. A rapid decline in elk density over the period of study in combination with fencing of hay storage yards (farm biosecurity), and non-selective culling were likely key factors resulting in a significant decline in *M. bovis* prevalence in elk between 2003 and 2014, and on overall decline in prevalence from 1997 for both species combined. The odds of a wild cervid testing positive for *M. bovis* through culture has declined approximately 94% since the late 1990's (1997 to 2000), when little or no bTB management was occurring. The average age of bTB positive wild elk has also increased

over time, indicating that transmission among younger age groups has declined or disappeared altogether. This study also provides precise estimates of important risk factors to further evaluate surveillance system effectiveness and probability of freedom in future.

Elk were the primary reservoir species for most of the period of study, but due to significant density reduction in combination with farm biosecurity measures to reduce artificial congregation, are now considered a spillover host in this ecosystem. White-tailed deer have likely always been a spillover host, primarily due to low densities, and if the ban on baiting and feeding of wildlife continues to be enforced, will likely remain that way in the forseeable future. Wild cervids should not be considered ideal hosts for *M. bovis* in North America but should be considered facultative hosts; acting either as a reservoir or spillover host dependent on both regional/local density and presence/absence of baiting and feeding.

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Table 4.1 Culture status, age category and sex of post-mortem sampled elk and white-tailed deer from GRME from 1997 to 2014

		Elk				WTD			
	Age Category	Female	Male	Unknown	Subtotal	Female	Male	Subtotal	Total
M bovis Negative	<1 year	361	150	1	512 (11.1%)	326	359	685 (7.17%)	1,197
	1-2 years	509	386	0	895 (19.4%)	620	2014	2634 (27.58%)	1,158
	3-5 years	1,162	767	2	1931 (41.8%)	1,190	4,091	5281 (55.29%)	721
	6-8 years	330	236	0	566 (12.3%)	111	258	369 (3.86%)	935
	8+ years	548	137	7	692 (15%)	52	397	449 (4.7%)	1,141
	Unknown	21	11	1	33 (0.7%)	22	111	133 (1.39%)	166
	Total	2931 (63.5%)	1687 (36.4%)	11 (0.06%)	4619	2321 (23.9%)	7230 (76.1%)	9,551	14,170
M bovis Positive	<1 year	1	0	0	1 (2.22%)	0	0	0 (0%)	1
	1-2 years	3	3	0	6 (13.33%)	1	1	2 (18.18%)	8
	3-5 years	4	7	0	11 (24.44%)	0	3	3 (27.27%)	14
	6-8 years	5	8	0	13 (28.89%)	0	6	6 (54.55%)	19
	8+ years	10	4	0	14 (31.11%)	0	0	0 (0%)	14
	Total	23 (51%)	22 (49%)	0	45	1 (9.1%)	10 (90.9%)	11	56

Table 4.2 Numbers of elk and white-tailed deer collected by various surveillance streams from 1997 to 2014 in the GRME.

	Elk					White-tailed Deer				
Year	Hunted	Opportunistic	Culled	Blood Test	Total	Hunted	Opportunistic	Culled	Blood Test	Total
9798	107	24	0	0	131 (2.8%)	0	4	0	0	4 (0%)
9899	265	7	0	0	272 (5.8%)	131	19	0	0	150 (1.6%)
9900	235	8	0	0	243 (5.2%)	93	27	0	0	120 (1.3%)
0001	526	23	0	0	549 (11.7%)	132	19	0	0	151 (1.6%)
0102	219	11	0	0	230 (4.9%)	521	14	0	0	535 (5.6%)
0203	422	9	0	39	470 (10.1%)	307	24	0	0	331 (3.5%)
0304	361	14	0	86	461 (9.9%)	347	13	226	0	586 (6.1%)
0405	244	8	0	52	304 (6.5%)	1,419	14	0	4	1437 (15%)
0506	309	11	0	19	339 (7.3%)	1,057	12	0	13	1082 (11.3%)
0607	235	1	0	33	269 (5.8%)	763	2	0	0	765 (8%)
0708	135	3	0	48	186 (4%)	590	4	0	10	604 (6.3%)
0809	116	4	47	17	184 (3.9%)	639	7	50	0	696 (7.3%)
0910	136	0	28	28	192 (4.1%)	610	13	0	0	623 (6.5%)
1011	297	9	0	65	371 (7.9%)	696	7	0	50	753 (7.9%)
1112	142	8	0	23	173 (3.7%)	676	23	0	36	735 (7.7%)
1213	159	6	0	28	193 (4.1%)	622	15	20	3	660 (6.9%)
1314	46	11	0	50	107 (2.3%)	277	3	0	50	330 (3.5%)
Total	3954 (84.6%)	157 (3.36%)	75 (1.6%)	488 (10.44%)	4674 (32.8%)	8880 (92.87%)	220 (2.3%)	296 (3.1%)	166 (1.74%)	9562 (67.2%)

Table 4.3 Logistic regression parameters from restricted model (includes elk density, n=8,025)

Variable	Factor level	Odds Ratio	Std. Err.	z	p > z	95% CI	
Year Category	1997/98 to 2000/01	Ref	Ref	Ref	Ref	Ref	Ref
	2001/02 to 2004/05	0.468	0.254	-1.400	0.163	0.162	1.358
	2005/06 to 2009/10	0.311	0.182	-2.000	0.046	0.099	0.979
	2010/11 to 2013/14	0.060	0.048	-3.490	0.000	0.012	0.290
Core	Core ref	16.252	8.318	5.450	0.000	5.960	44.316
Sex	Male ref	1.869	0.564	2.070	0.038	1.035	3.376
Age Category	< 1 year	Ref	Ref	Ref	Ref	Ref	Ref
	1-2 years	2.310	2.492	0.780	0.438	0.279	19.135
	3-8 years	3.413	3.531	1.190	0.236	0.449	25.935
	9+ years	8.638	9.201	2.020	0.043	1.071	69.682
Species	Elk ref	1.022	0.456	0.050	0.962	0.426	2.451
Surveillance Method	Hunt	Ref	Ref	Ref	Ref	Ref	Ref
	Opportunistic	3.009	2.181	1.520	0.129	0.727	12.458
	Culled	2.116	1.353	1.170	0.241	0.605	7.408
	Blood Test	6.715	3.589	3.560	0.000	2.355	19.142
Elk Density		3.047	1.372	2.470	0.013	1.261	7.363
intercept		0.000	0.000	-7.170	0.000	0.000	0.002

Ref – reference category for factor variables.

Table 4.4 Logistic regression parameters from unrestricted model (not including elk density, n=14,060)

Variable	Factor level	Odds Ratio	Std. Err.	z	p > z	95% CI	
Year Category	1997/98 to 2000/01	Ref	Ref	Ref	Ref	Ref	Ref
	2001/02 to 2004/05	0.406	0.219	-1.670	0.094	0.141	1.168
	2005/06 to 2009/10	0.237	0.137	-2.490	0.013	0.076	0.737
	2010/11 to 2013/14	0.031	0.024	-4.500	0.000	0.007	0.140
Core	Core ref	22.432	10.591	6.590	0.000	8.891	56.593
Sex	Male ref	1.930	0.573	2.220	0.027	1.079	3.452
Age Category	< 1 year	Ref	Ref	Ref	Ref	Ref	Ref
	1-2 years	2.447	2.639	0.830	0.407	0.296	20.253
	3-8 years	3.388	3.505	1.180	0.238	0.446	25.736
	9+ years	8.181	8.726	1.970	0.049	1.011	66.172
Species	Elk ref	0.711	0.301	-0.810	0.420	0.309	1.632
Surveillance Method	Hunt	Ref	Ref	Ref	Ref	Ref	Ref
	Opportunistic	4.971	3.374	2.360	0.018	1.314	18.804
	Culled	4.055	2.396	2.370	0.018	1.274	12.912
	Blood Test	15.279	7.025	5.930	0.000	6.205	37.625
intercept		0.000	0.000	-7.200	0.000	0.000	0.002

Ref – reference category for factor variables.

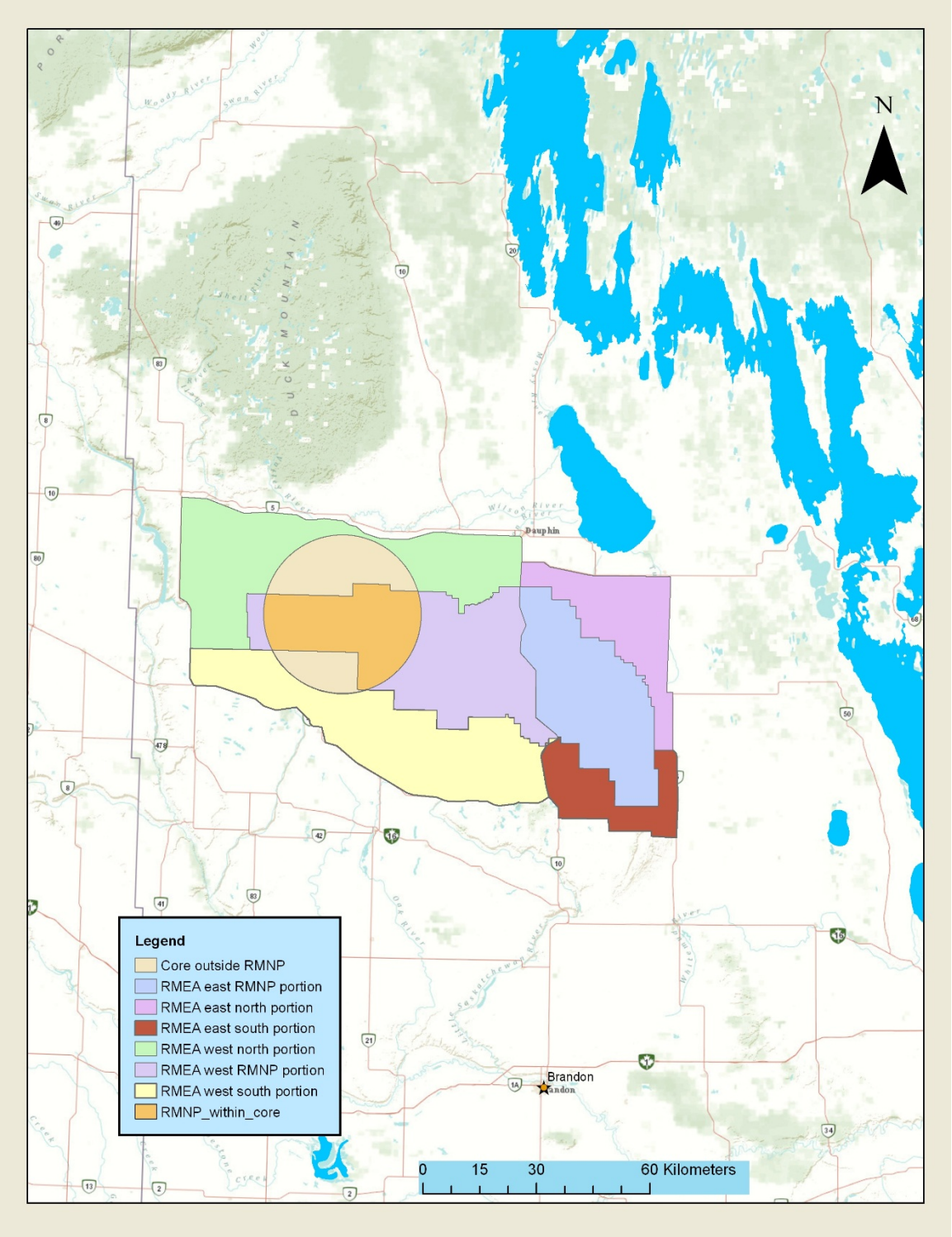

Figure 4.1 Subzones within the Riding Mountain Eradication Area (RMEA) for estimating elk density.

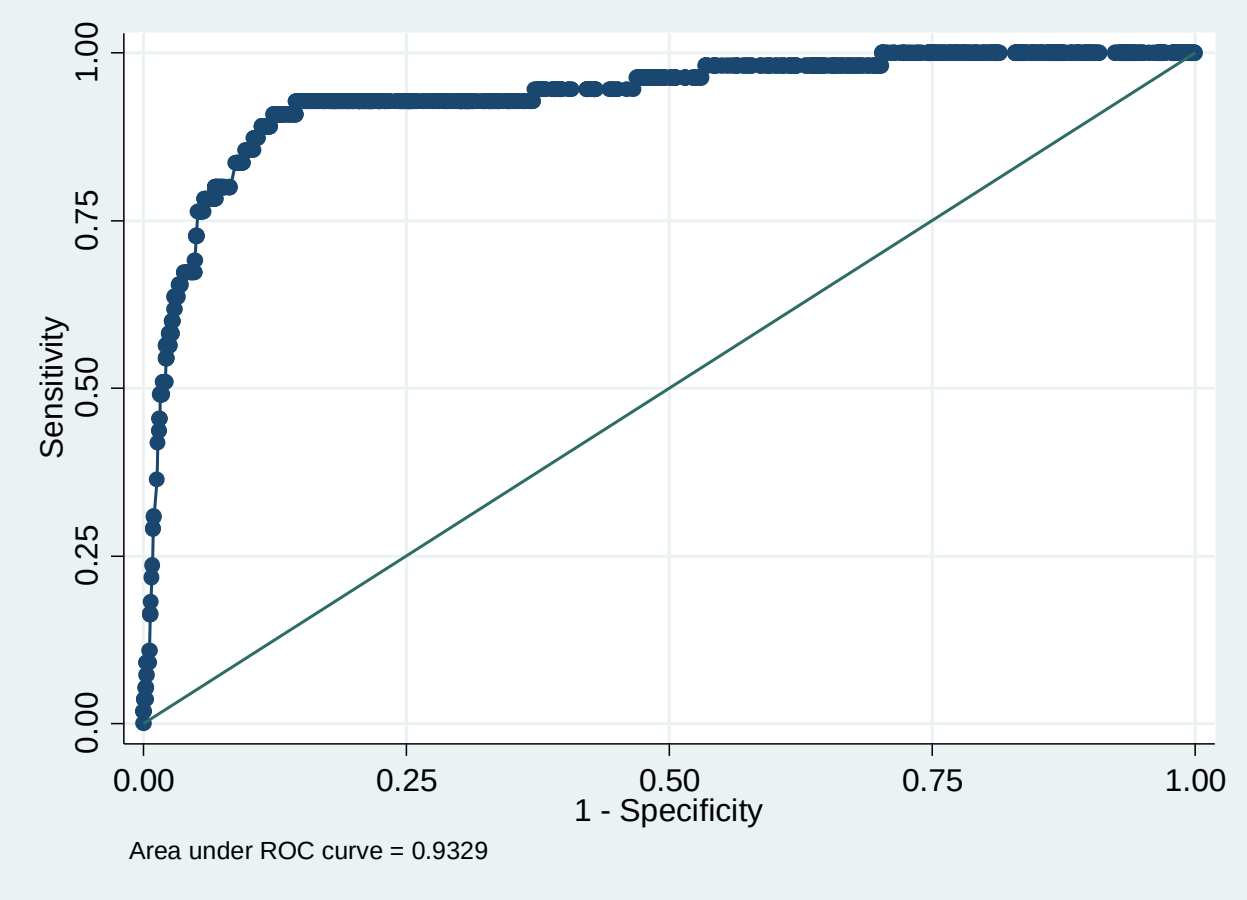

Figure 4.2 ROC graph of restricted logistic regression model

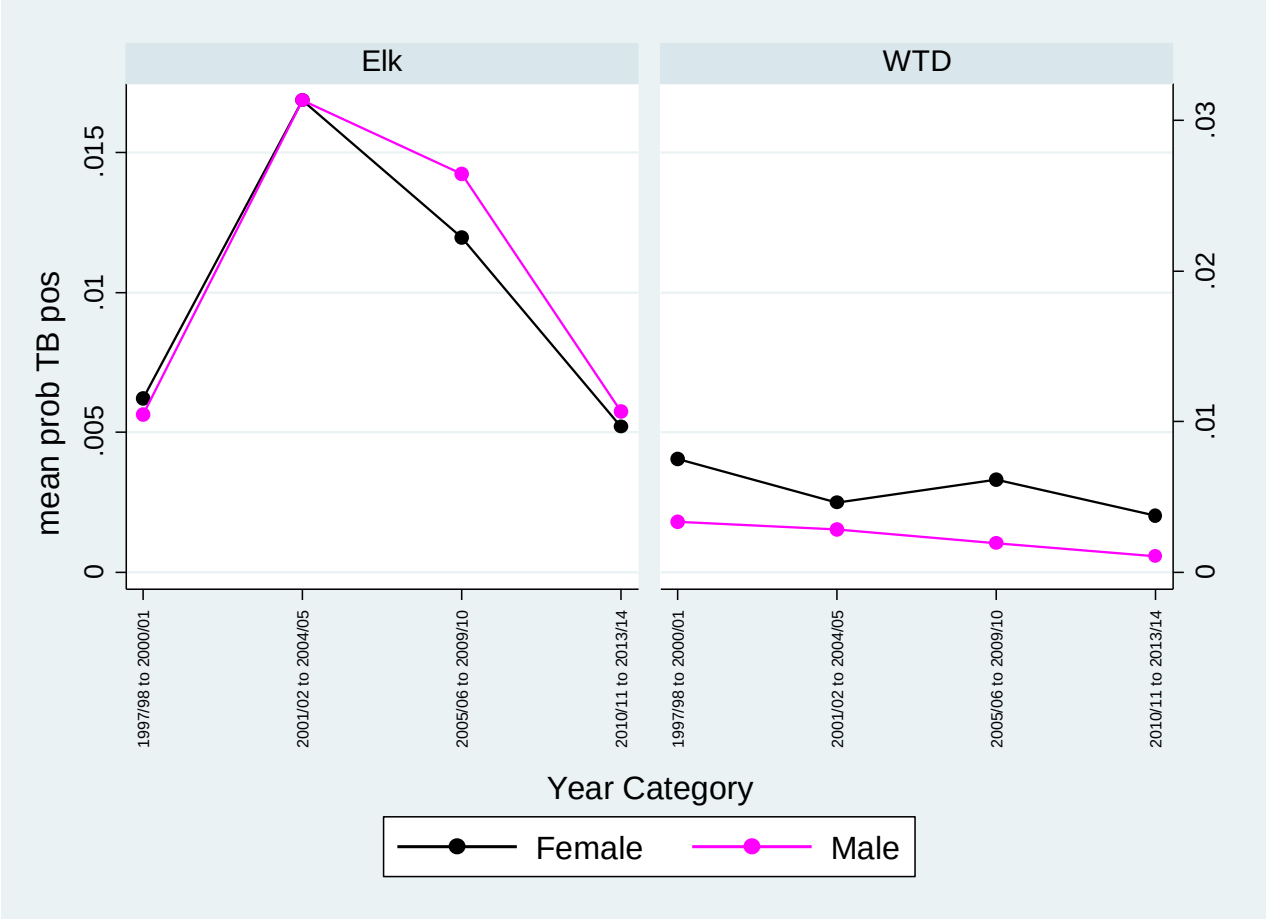

Figure 4.3 Mean probability of being culture positive for elk and WTD by sex by year category.

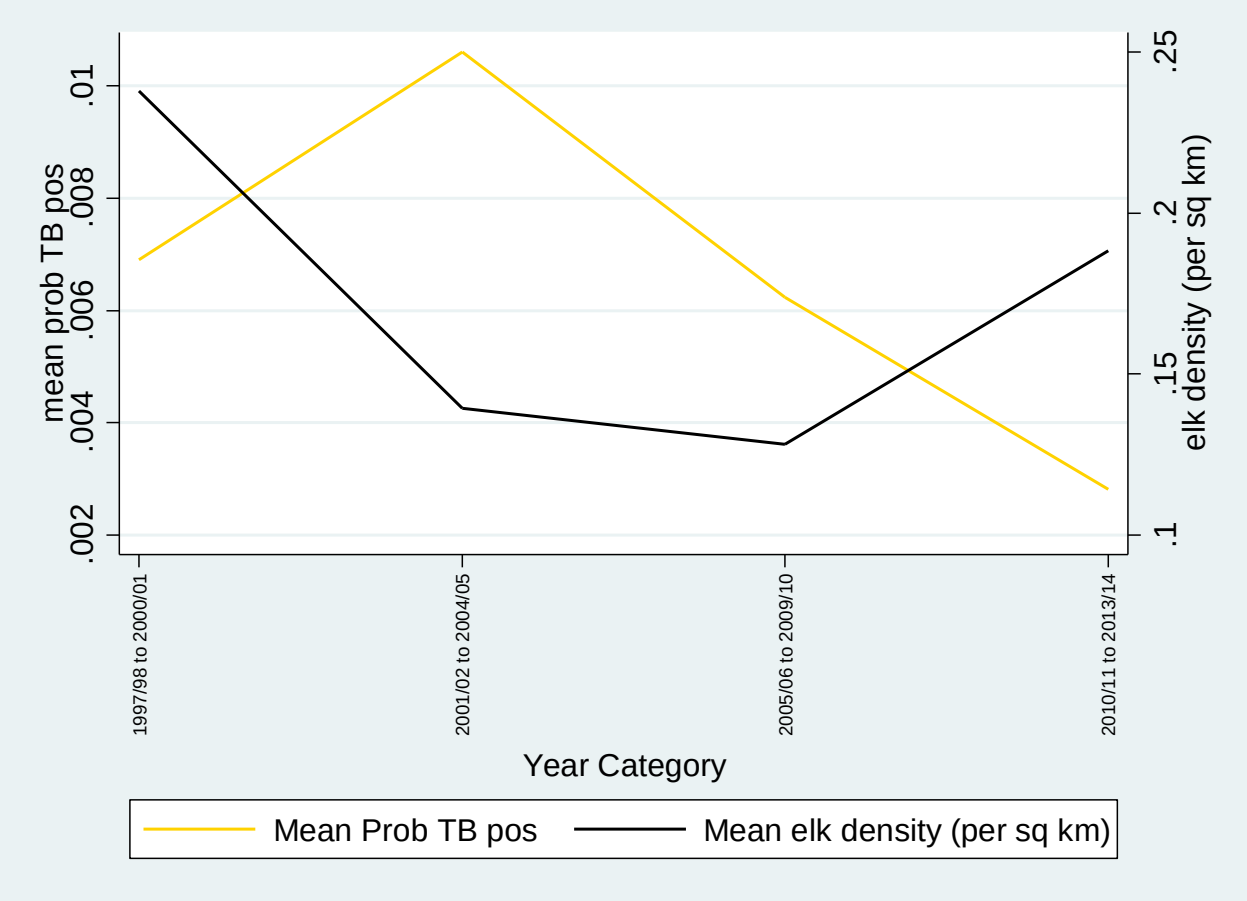

Figure 4.4 Elk density versus mean probability of being culture positive from restricted model by year category.

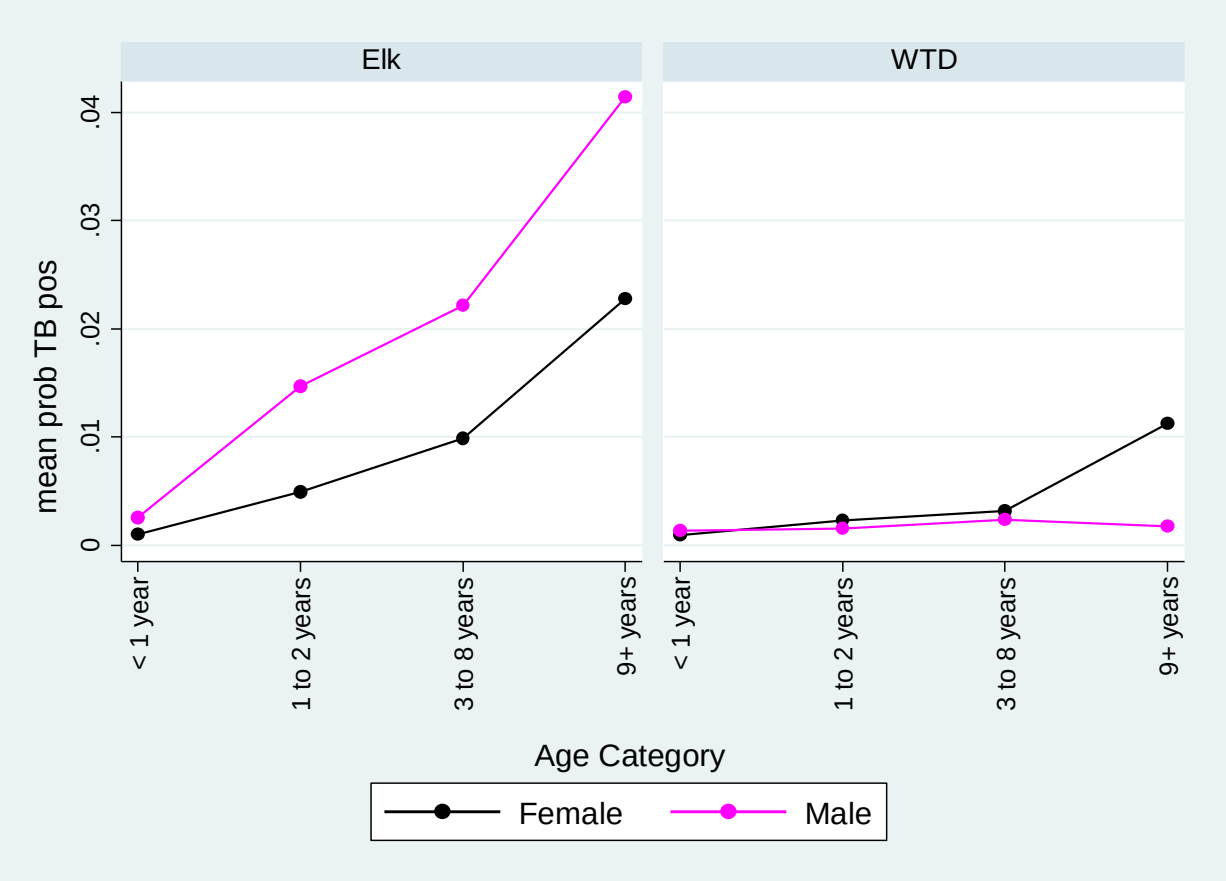

Figure 4.5 Age category versus mean probability of being culture positive for male and female elk and white-tailed by year category using restricted model.

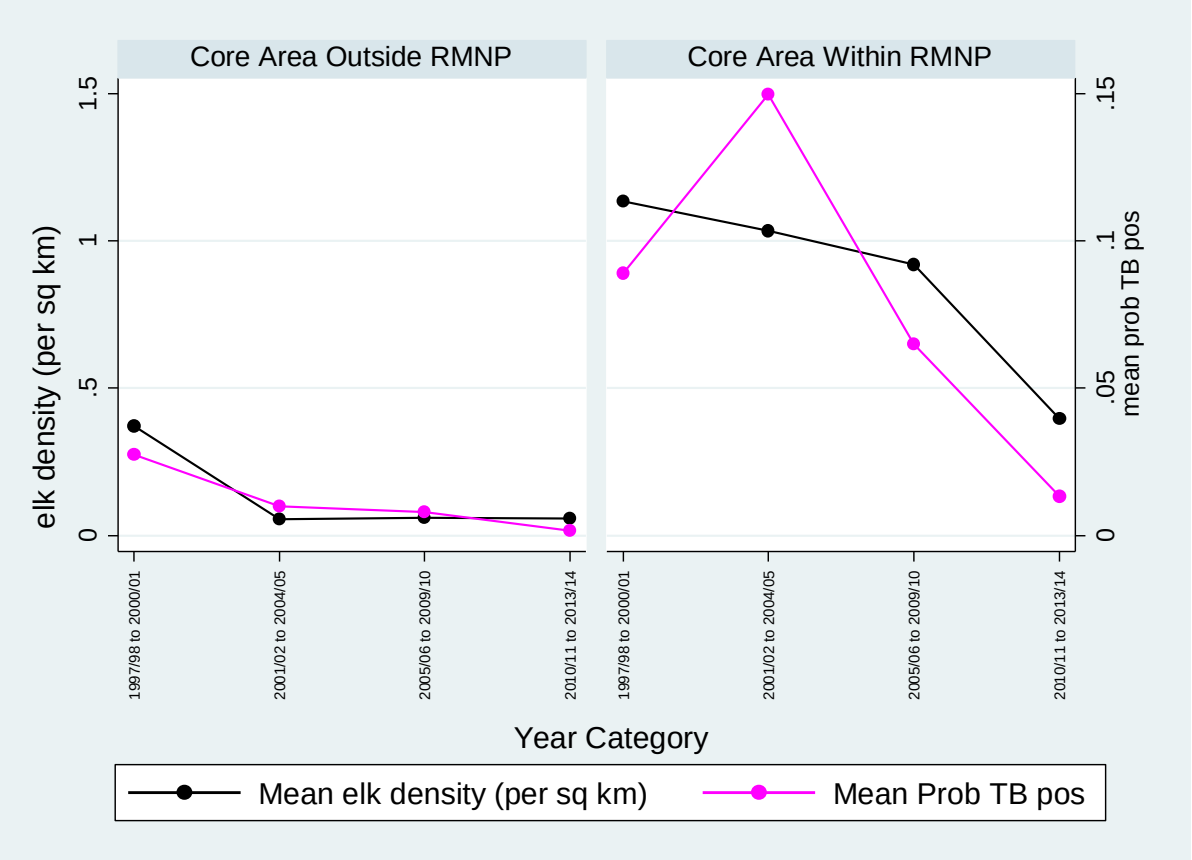

Figure 4.6 Elk density and probability of being culture positive by year category within the core area inside and outside RMNP 1997 to 2014.

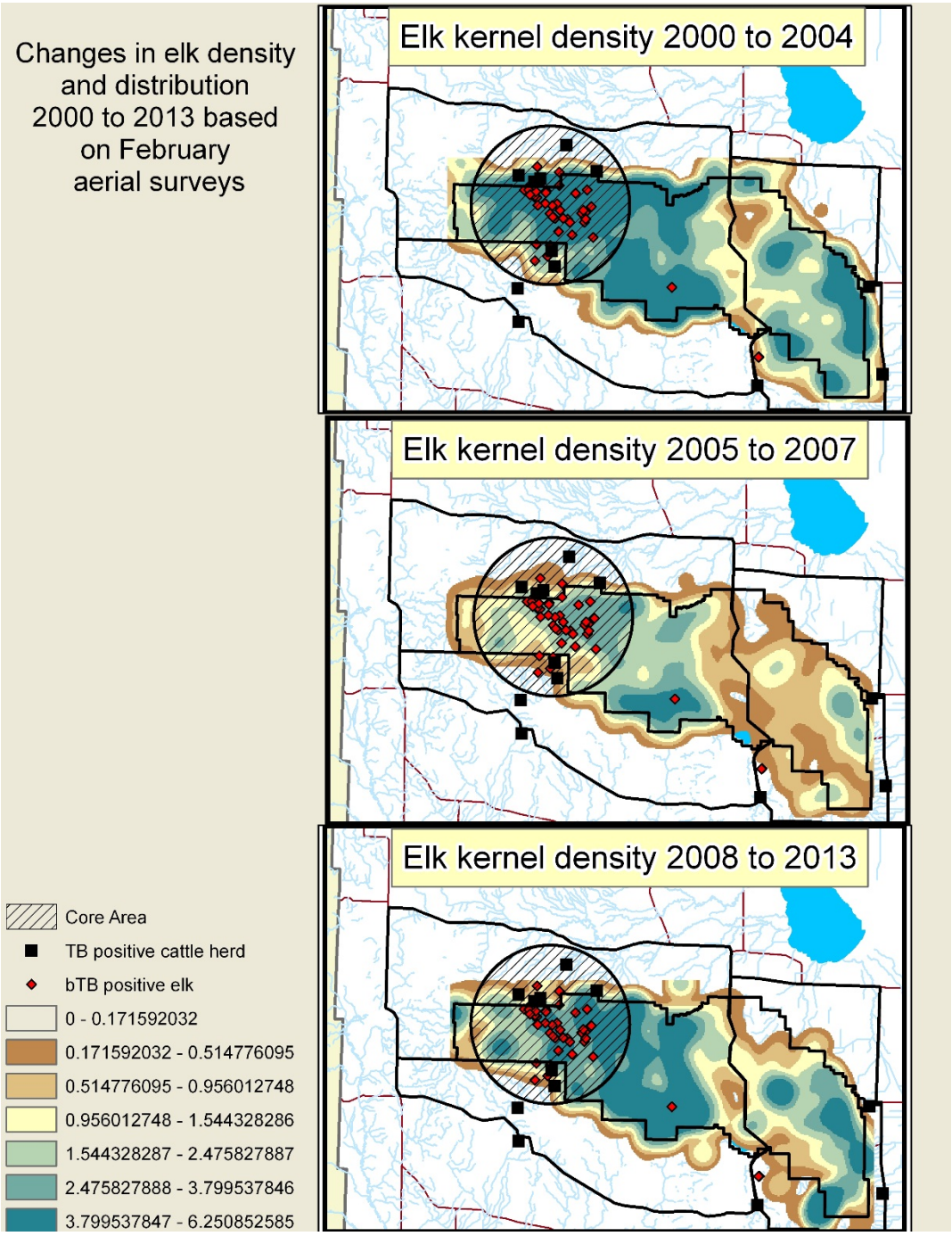

Figure 4.7 Temporal changes in elk density (spatial kernel) between 2000 and 2013 within RMNP with bTB positive elk and cattle herds

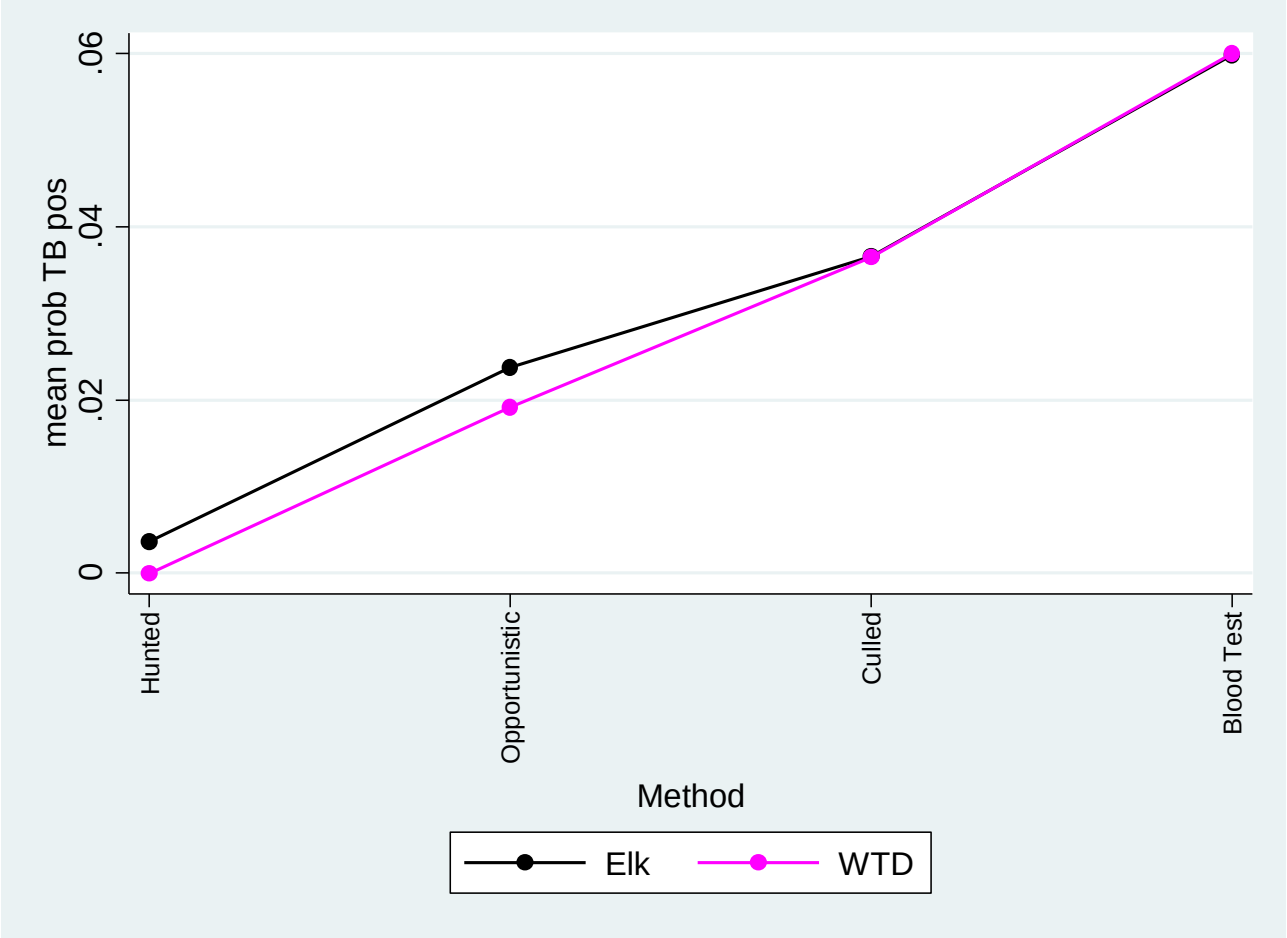

Figure 4.8 Method of surveillance versus probability of being culture positive for elk and white-tailed deer using the restricted model.

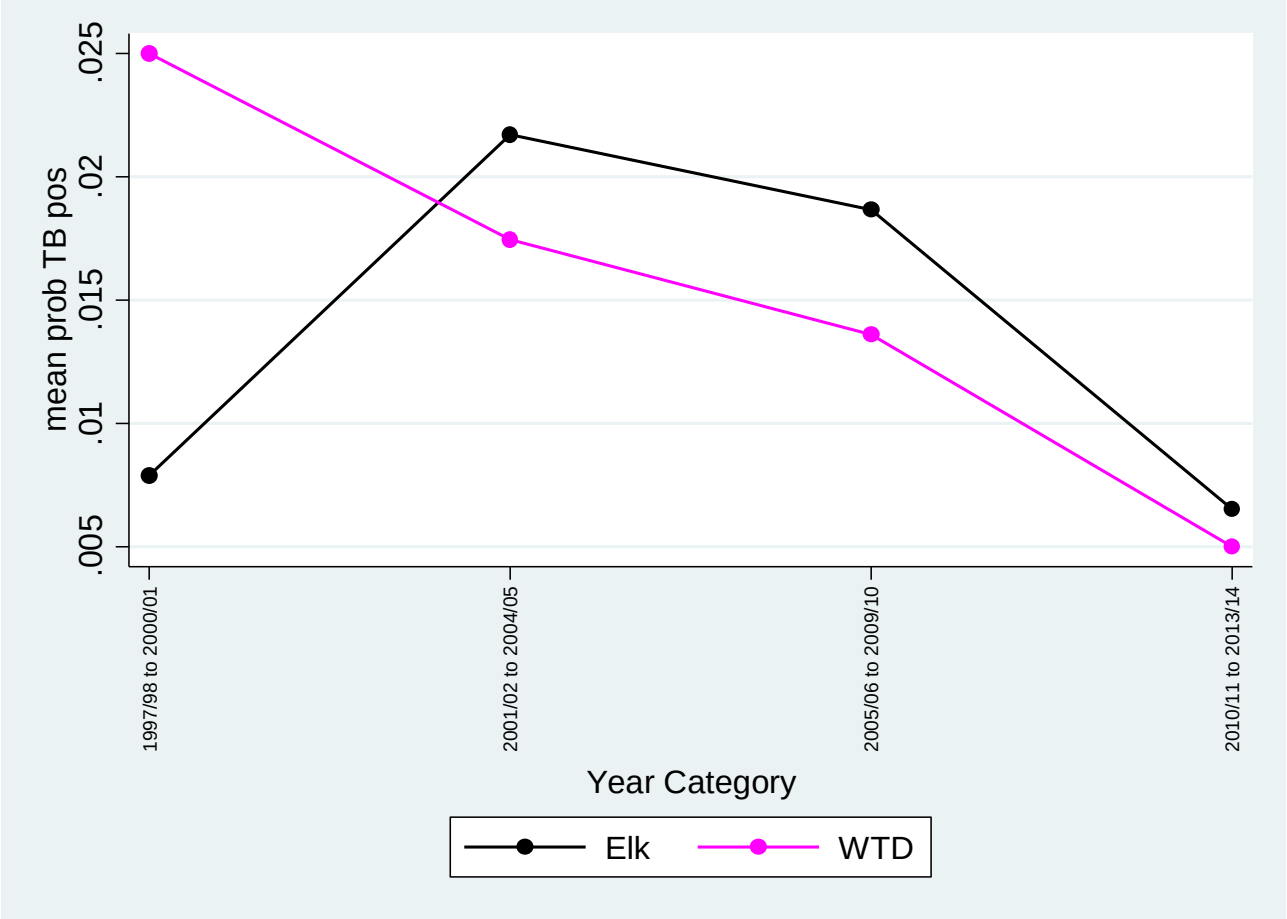

Figure 4.9 Year of surveillance versus probability of being culture positive for elk and white-tailed deer by sampling year (biological year from June 1st to May 30th) from 1997 to 2013.

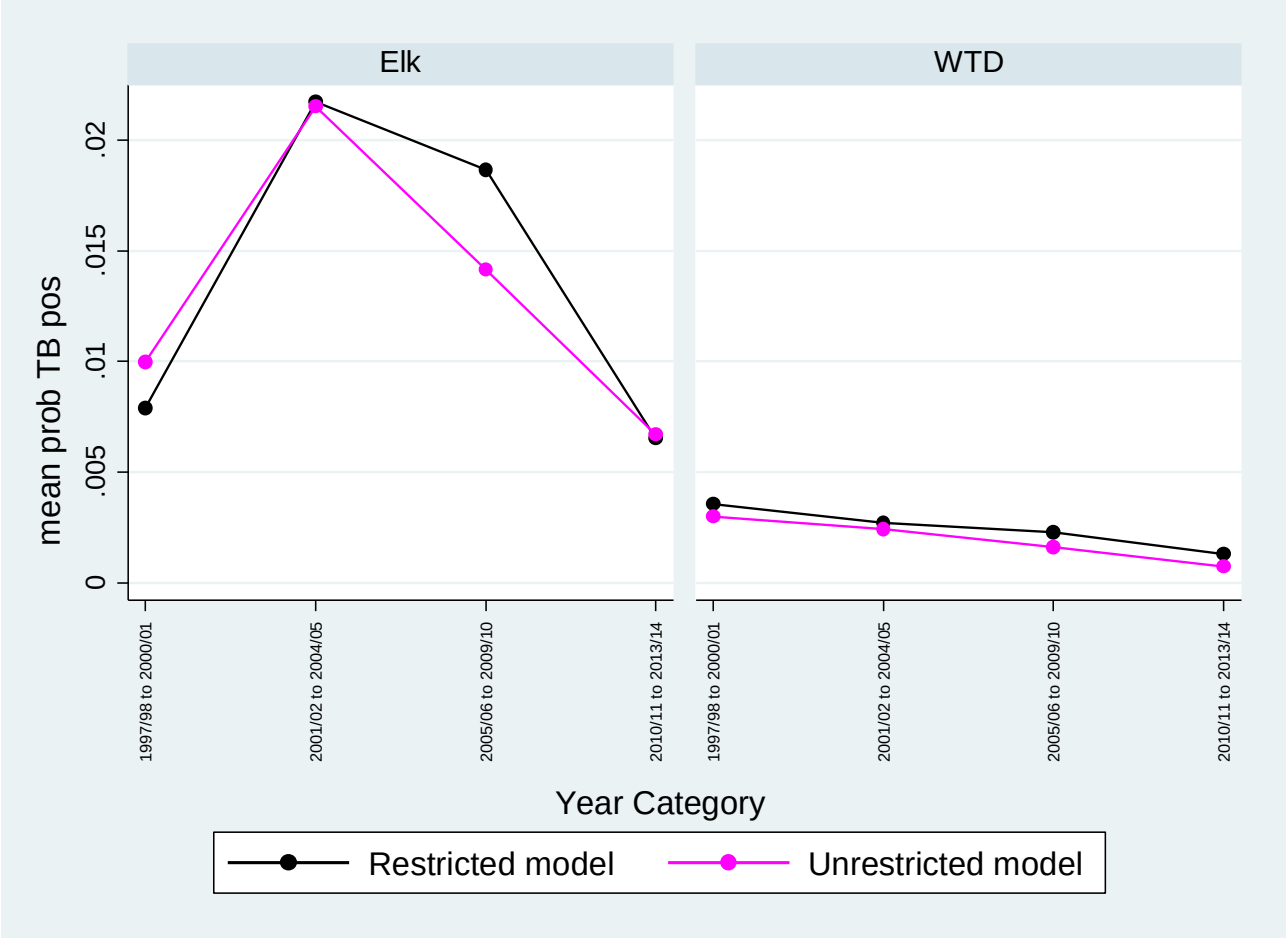

Figure 4.10 Year category versus probability of being culture positive for restricted and unrestricted models by species from 1997 to 2014.

Figure 4.11 Median (horizontal bar) and interquartile range (boxes, bars are upper and lower adjacent values) of cementum age of culture positive elk and white-tailed deer from by sampling year (biological year from June 1st to May 30th) 1998 to 2014.

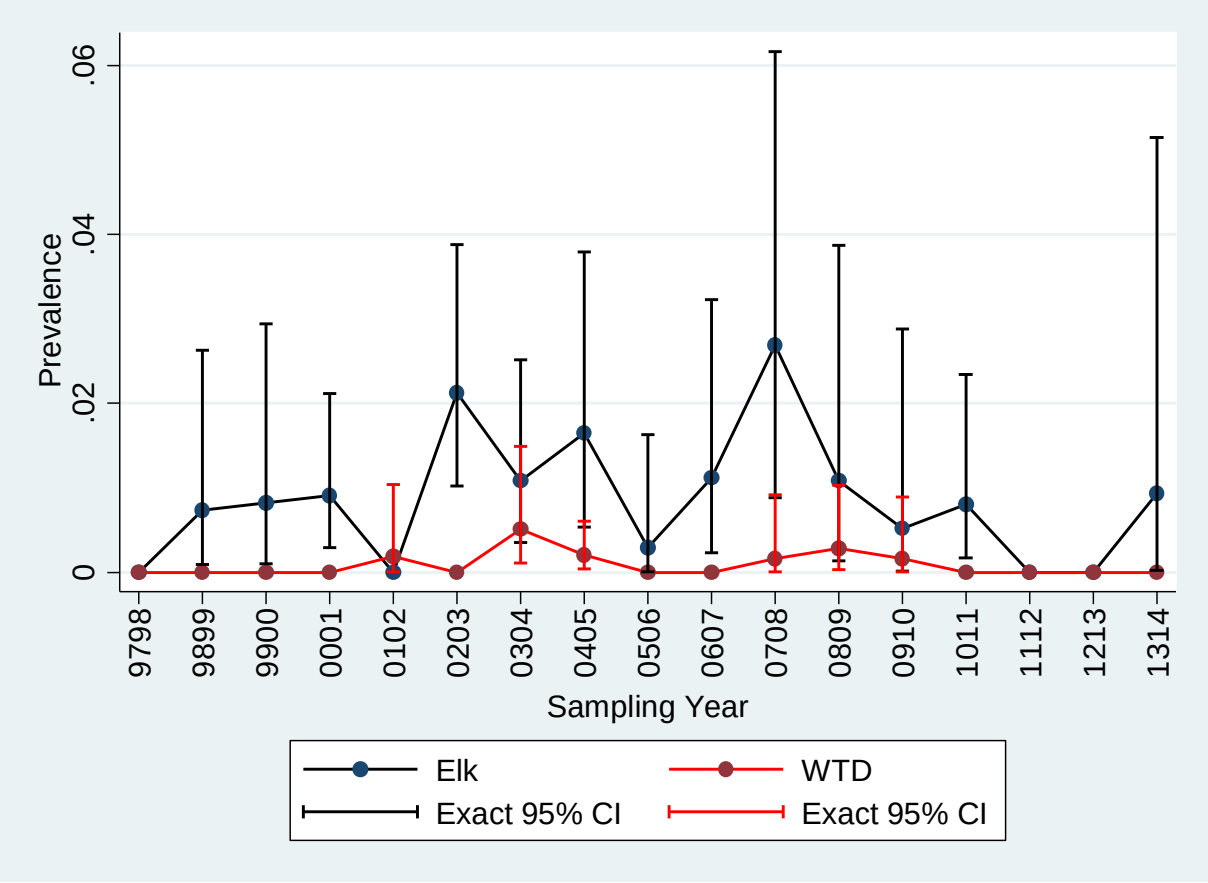

Figure 4.12 Annual *M. bovis* prevalence (bars represent exact 95% confidence interval) in the Riding Mountain Eradication Area (RMEA) for elk and white-tailed deer by sampling year (biological year from June 1st to May 30th) from 1997 to 2014.

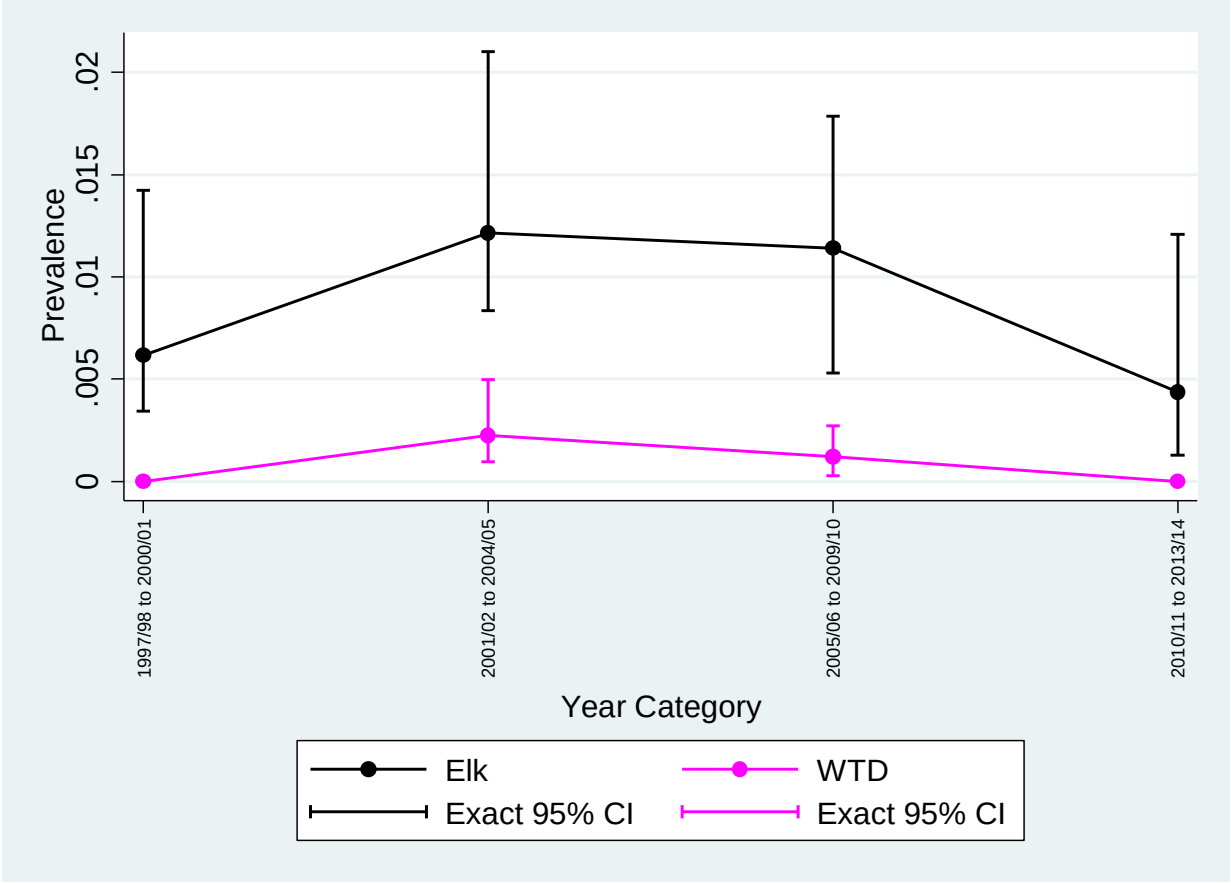

Figure 4.13 *M. bovis* prevalence by year category (management phase) for elk and white-tailed deer from 1997 to 2014 with exact 95% confidence intervals.

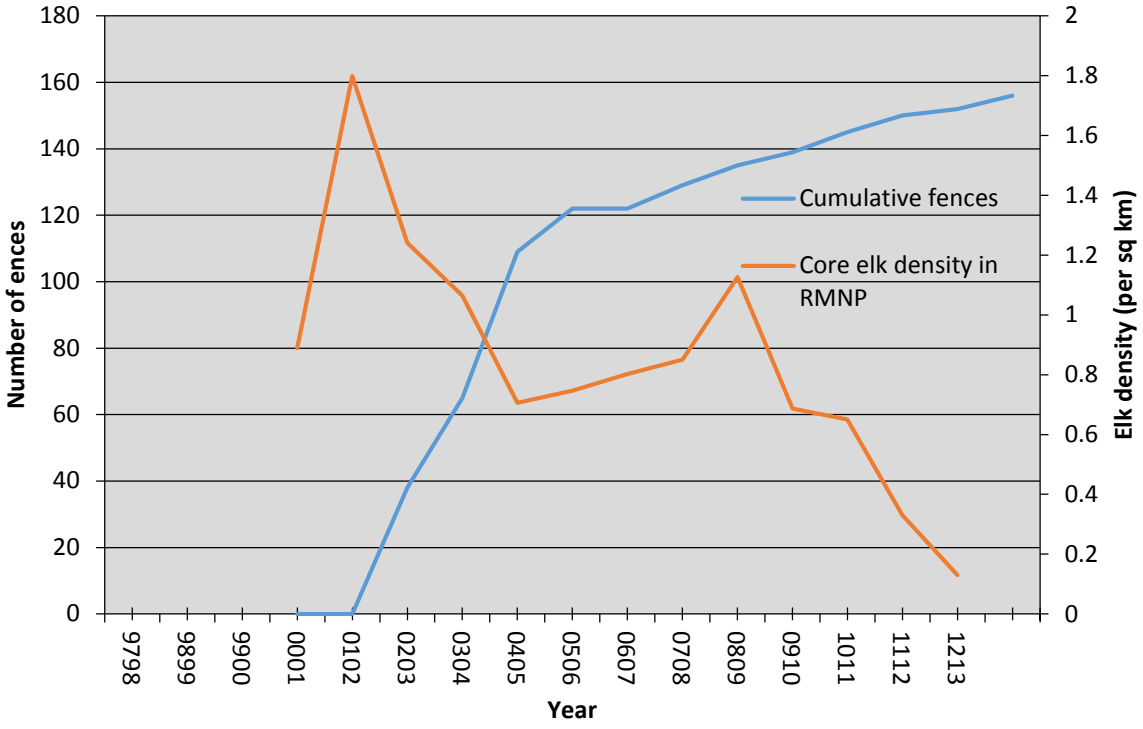

Figure 4.14 Cumulative hay barrier fences constructed and elk density per year within the core area by sampling year (biological year from June 1st to May 30th) from 1997 to 2013.

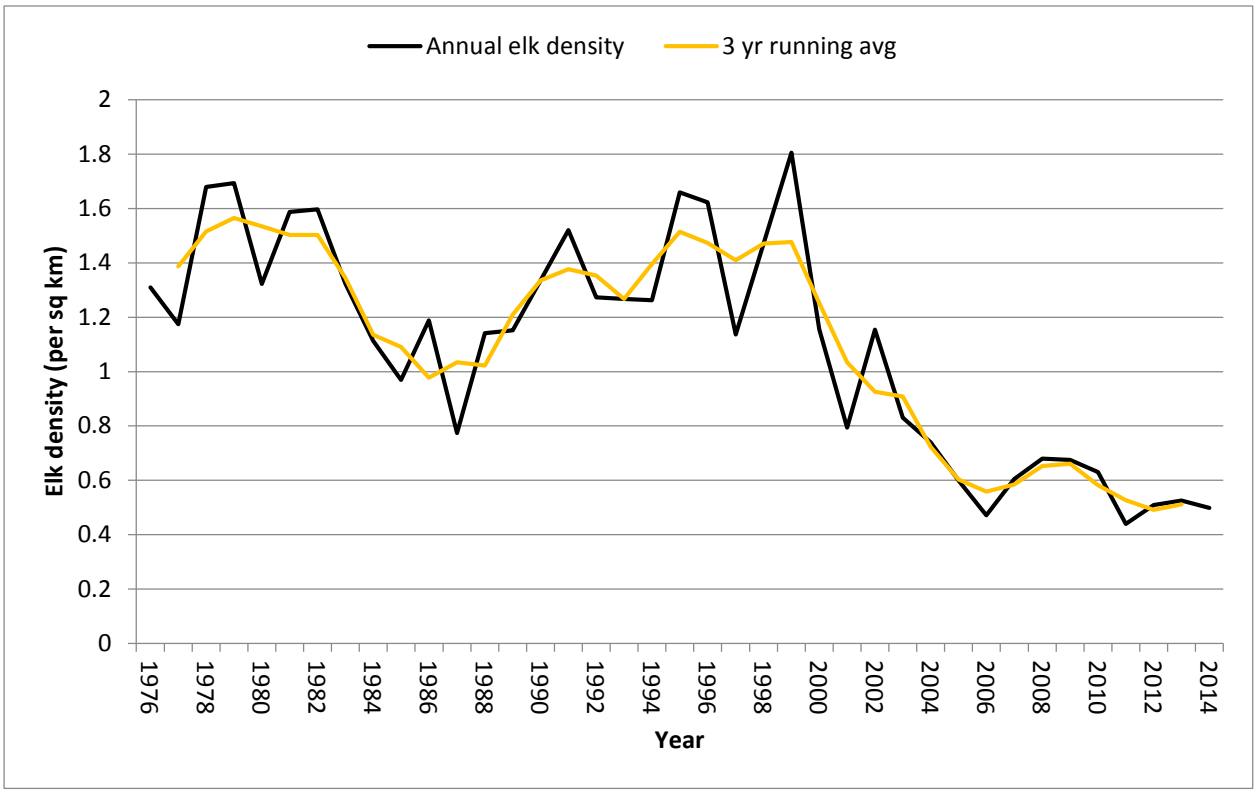

Figure 4.15 Elk density (annual and 3 yerar running average) within Riding Mountain National Park by sampling year (biological year from June 1st to May 30th) from1976 to 2011.

CHAPTER 5: SPATIAL AND MOLECULAR EPIDEMIOLOGY OF *MYCOBACTERIUM BOVIS* IN THE GREATER RIDING MOUNTAIN ECOSYSTEM

While the previous chapters provide an understanding of the risk factors and validity of blood testing of M. bovis overall, this chapter provides a more detailed understanding of the different genotypes of M. bovis that have been isolated from the three host species and how they are related spatially and temporally. This is important to understand transmission pathways and how the organism has likely changed over time, where it likely originated and how it is related to other M. bovis genotypes in North America and worldwide. The research presented here builds on previous research by providing greater genotypic resolution for this localized outbreak as well as providing a solid scientific basis for where future disease management should be directed.

5.1 Introduction

The advancement of molecular diagnostic techniques to characterize mycobacterial outbreaks has revolutionized epidemiological research and revealed previously unknown transmission pathways for members of the *Mycobacterium tuberculosis* complex (MTC), including *M. bovis* and *M. tuberculosis* (Skuce et al., 2002; Skuce et al., 2005; Martinez et al., 2008; Milian-Suazo et al., 2008; Michel et al., 2009; Humblet et al., 2010; Muellner et al., 2011; Parreiras et al., 2012). These techniques have also allowed much better understanding of spatial patterns of transmission between wildlife reservoirs and domestic species, allowing disease management and control techniques to become more targeted and efficient (Kazwala et al., 2006; Michel et al., 2008; Naranjo et al., 2008; Romero et al., 2008; ÁLvarez et al., 2009; Boniotti et al., 2009; Michel et al., 2009; Moonan et al., 2009; Biffa et al., 2010). Common bacterial typing techniques used to identify most bacterial genomes such as multilocus sequence typing (MLST) are generally not effective with most mycobacterial species (Pitondo-Silva et al., 2013), due to their close relatedness (99.9% nucleotide identity) and similarity of 16S ribosomal RNA sequences (Smith et al., 2006a; Smith, 2012). Most rapid typing schemes for *M. bovis* rely on polymerase chain reaction (PCR) amplification of loci within repetitive regions of DNA which effectively discriminate different strain types. Prior to this, restriction fragment polymorphism (RFLP) and restriction endonuclease (REA) techniques provided high discriminatory ability with most

mycobacterial isolates, but were very labour intensive, relatively slow, and required large amounts of DNA (Cousins et al., 1998; Christianson et al., 2010). Additionally, most *M. bovis* isolates do not contain adequate copy number of IS6110 for high resolution RFLP typing, making it less useful.

Spoligotyping is a relatively rapid typing technique that uses PCR to amplify a polymorphic region of the *M. bovis* genome called the direct repeat (DR) region; this is the standardized technique used by the Canadian Food Inspection Agency (CFIA) to type mycobacterial isolates from domestic and wild animals in Canada. It has been used to elucidate spatial patterns and epidemiological links in several wildlife reservoirs around the globe (Martinez et al., 2008; Milian-Suazo et al., 2008; Romero et al., 2008; Zanella et al., 2008a; Cross et al., 2009; Michel et al., 2009; Santos et al., 2009; Rodwell et al., 2010).

Recurring outbreaks of bovine tuberculosis have been discovered in elk, cattle and white-tailed deer since the early 1990's in an area surrounding Riding Mountain National Park (Lees et al., 2003; Lees, 2004) designated the Greater Riding Mountain Ecosystem (GRME). Previous research using both spoligotyping and RFLP has shown that elk and cattle isolates from the GRME were very closely related (Cousins et al., 1998; Lutze-Wallace et al., 2005b) and that an *M. bovis* isolate from a wolf found dead within Riding Mountain National Park in 1978 was the same spoligotype currently found in elk and cattle (Lutze-Wallace et al., 2005a). Two spoligotypes, designated MB-1 (SB1070) and MB-2 (SB1071), which vary at only one of 43 loci within the DR region, have been found in both elk and cattle, which apparently share both spoligotypes. Further refinements in other mycobacterial typing techniques such as multiple locus variable number of tandem repeat analysis (MLVA), have allowed better discrimination of MTC isolates, especially when combined with spoligotyping. MLVA, which uses differences in

mycobacterial interspersed repetitive units (MIRU) at specific variable loci using 15 or 24 loci, provides similar levels of discrimination to RFLP techniques at regional and local scales (Allix-Beguec et al., 2008; Jagielski et al., 2014).

The objective of the current research was to use 24 locus MLVA typing in combination with spoligotyping to further discriminate banked *M. bovis* isolates isolated since 1990 from elk, deer and cattle from the GRME. These data were then used to further understand the epidemiological relationships between these three host species, as well as to look at overall genotypic diversity over this period. A second objective was to undertake a preliminary analysis of spatial patterns of occurrence of *M. bovis* and specific genotypes within the GRME to better characterize this outbreak.

5.2 Methods & Materials

Preliminary exploratory analysis was carried out using the spatial scan statistic (Kulldorff, 1997) to determine location and size of any spatial clusters of *M. bovis* isolates in elk and WTD. Elk are considered the primary wildlife reservoir of *M. bovis* in this region (Lees et al., 2003; Nishi et al., 2006; Shury and Bergeson, 2011; Fitzgerald and Kaneene, 2013), with white-tailed deer likely being a spillover host, so the primary risk to surrounding cattle herds from infected wildlife depends primarily on the spatial location of these two free-ranging species. A preliminary analysis used all elk and WTD locations at time of euthanasia from *M. bovis* culture positive (case) and negative (control) animals sampled from hunter killed, opportunistic, and blood testing surveillance streams (Shury and Bergeson, 2011). Initial analysis was carried out using SatScan v 9.1.1. This analysis used a space-time Bernoulli model with both circular and elliptical clusters with maximum spatial cluster size of 50% of the population at risk, restricted to areas of high rates with no geographical overlap of clusters without consideration for genotype (using all *M. bovis* isolates from elk and WTD). Cattle were not included in this model as they

were clustered by herd and surveillance was targeted to herds with epidemiological links (traceback and traceout herds).

Further exploratory analysis using SatScan to determine spatial clusters of different miruspoligotypes using a multinomial model restricted to *M. bovis* positive isolates with confirmed miruspoligotypes (n=68) from elk, deer and cattle was also carried out with a maximum temporal window restricted to 30% of study period. A hotspot analysis to determine spatial clustering of high prevalence areas was also conducted using ArcGIS 10.2 (ESRI, Redlands, CA) by creating a spatial weights matrix and setting the distance band to 6 kilometre maximum.

Seventy different *M. bovis* isolates, initially isolated between 1992 and 2011, were available to be characterized by spoligotyping and MLVA typing. These isolates were obtained from the Mycobacterial Diseases Centre of Excellence (MDCE) laboratory (Ottawa, Ontario, Canada) where they had been archived and frozen at -80 °C. Included were 18 cattle isolates (25.7%) representing 13 different herds, designated A through M, 42 elk isolates (60%) and 10 white-tailed deer isolates (14.3%). Cultures were revived by inoculating an aliquot into 7H9 broth culture and incubating for 3 to 4 weeks at 37 °C. DNA was extracted using the methods of (Skuce et al., 1994). 24 locus MIRU-VNTR typing was performed at the National Reference Centre for Mycobacteriology (Winnipeg, Manitoba, Canada) using the methods outlined in (Christianson et al., 2010). Allelic diversity was calculated using formula described in Hunter and Gaston (1988).

5.3 Results

5.3.1 Molecular results

Of the *M. bovis* isolates isolated from cattle, deer and elk in the GRME between 1990 and 2011, only three elk and one white-tailed deer isolates were not available for genotyping (5.4%). Two isolates (one cattle and one white-tailed deer) had MIRU-VNTR loci that would not amplify, despite repeated attempts and so data from these two isolates were not used for further analyses, leaving a total of 68 *M. bovis* isolates that were successfully genotyped. Two spoligotypes (SB1070 & SB1071), which only varied at oligonucleotide 12, and two different MIRU-VNTR types, which only varied at a single locus (2165, ETR-A) were found within this set of isolates.

The allelic diversity index for the single variable locus (2165) was 0.24, while all other 23 MLVA loci were monomorphic among the 68 isolates examined. One spoligotype (SB1070) has only been found in cattle and was restricted to an outbreak that occurred among 5 infected herds during the early 1990's (Munroe et al., 1999). A total of 4 miruspoligotypes were classified through combinations of these two typing systems. Only one MLVA (type 3), was found in all three host species, and this was also the only type found in white-tailed deer, and was the most common genotype. The clustering rate for the MLVA typing method was 0.971 and for the spoligotyping method was 0.957.

Miruspoligotype 4 demonstrated strong temporal clustering within infected elk while strain type 3 did not (Figure 1). Four infected elk with genotype 4 were detected between 2000 and 2003 and it was not detected again until four elk were again detected in 2008 and 2009. The latter four elk ranged in age from 5 to 17 years of age. Only single isolates were available for 9 of 12 cattle herds genotyped, while the other three all shared identical miruspoligotypes, with two to 5 isolates available per herd. Trace-in and trace-out investigations completed by CFIA

district veterinary staff indicated that in 9 of 12 cattle herds, transmission was due either to latent reactivation from prior exposure or from exposure to wildlife (pers. comm. L. Bates, CFIA). This was clearly evident in the case of one cattle herd in the RM of Grandview in which miruspoligotype 4 was genotyped from a farm with a single infected bull from a beef herd in the same winter as a subadult (3 to 5 year old) bull elk harvested within 4.6 km of this farm. Only one infected animal was found in this herd during depopulation and no subsequent trace-out or trace-backs were discovered, indicating that infection was likely acquired locally in the environment. In most cases, elk were either observed co-mingling with cattle or feeding on hay bales left in fields during epidemiological tracing investigations.

5.3.2 Spatial analysis

The initial exploratory analysis which examined all *M. bovis* isolates revealed a single most likely cluster ($p < 0.001$) centered in the west side of RMNP near the north boundary. This circular cluster contained 46 of 55 *M. bovis* culture positive elk and white-tailed deer discovered through annual surveillance between 1992 and 2011 and the relative risk of being culture positive was 39.8 ($p < 0.001$), compared to outside this area. A similar elliptical cluster centered on the same area, but only encompassed 42 of the 55 positive animals. This circular configuration with the addition of a 10 km radius buffer to account for uncertainty in home range of elk was later adopted as a Core Area where disease management activities were concentrated by the Scientific Review Committee (SRC) of the Manitoba Bovine TB Task Force (pers. Comm. P. Paquet, chair of RMNP SRC).

The multinomial analysis revealed a single most likely spatial cluster consisting of four elk with miruspoligotype 4 (Figure 5.2). Three of these were bull elk collected by blood sampling between 2003 and 2008, and the fourth was a 17 year old cow elk also collected through blood

sampling. A second cluster was identified that was marginally significant (p=0.065) consisting of two cattle herds with miruspoligotype 1 isolated in 1991.

The Getis-Ord hotspot analysis revealed several clusters around individual bTB positive elk or deer, but a main cluster in the northwest portion of RMNP which matches the designated Core Area relatively closely (Figure 5.3)

5.4 Discussion

Very little diversity exists within the *M. bovis* isolates from the GRME, with only two spoligotypes and two MLVA types found in 68 isolates over a 22 year period. As reported previously (Lutze-Wallace et al., 2005b), variation in the two spoligotypes was restricted to deletion of a spacer at oligonucleotide 12, and no other variations in spoligotypes were confirmed from this set of isolates for this outbreak. Variation in MIRU-VNTR type also only occurred at a single locus, 2165 (ETR-A), as either 8 or 9 copies. The extremely limited genotype diversity found within this set of isolates suggests one of two likely scenarios; 1) that there was only a single introduction event into this wildlife reservoir with repeated spillback events into surrounding cattle herds since 1990, or 2) that this was the only genotype to persist in this wildlife reservoir and that other genotypes were present, but not sampled and effectively disappeared over time.

O'Connor et al (2012) describe the epidemiological system that maintains *M. bovis* in wildlife reservoirs as an episystem. The protracted outbreak described herein is a relatively simple episystem consisting of only one major reservoir species (elk) and two non-maintenance or spillover hosts (cattle and WTD) with very limited strain diversity. Other similar wildlife reservoirs typically have more diverse genotypes using similar typing methodology. In Doñana National Park in Spain, eight different spoligotypes and eight different MIRU-VNTR profiles have been described using only 8 VNTR loci (Romero et al., 2008). The host diversity within

this Spanish wildlife reservoir is also greater, including red deer, fallow deer, wild boar and cattle. In the Republic of Ireland, a combination of MLVA and spoligotyping discriminated 54 different profiles using only 6 MLVA loci from a large set of isolates (n=386) from badgers, cattle and red deer (McLernon et al., 2010).

Considering the moderate number of isolates examined and the large number of MLVA loci examined in the GRME, there is remarkably little diversity within the mycobacterial isolates from three different host species over a two decadal span of time. This is consistent with a clonal expansion of a limited introduction, with no importation of new cases or strains (Gardy et al., 2011). While 24 loci MLVA provides good discriminatory power for MTC isolates, whole genome sequencing provides much greater discrimination in clonal outbreaks and additional transmission chains may become evident using this technique (Ford et al., 2012; Roetzer et al., 2013).

The temporal distribution of the four miruspoligotypes differs somewhat between species. The SB1070 spoligotype was only found in cattle in the outbreak that involved five cattle herds in 1990/91 (Figure 5.1). The two miruspoligotypes associated with this spoligotype (1 and 2) were only isolated from cattle in this period and were never found among wildlife isolates or later cattle isolates. The only existing wildlife isolate from this time period, which was from the index wild elk found to be infected in this area, was of a different miruspoligotype. This particular elk was a hunter killed bull harvested as part of a special hunt in October of 1992 only 5 kilometres from the farm considered the source of the cattle outbreak in 1990/91. This elk isolate differed in spoligotype, but had the same MLVA type as the cattle isolates. It is likely that this cattle outbreak was associated with contact with infected elk, even though the spoligotypes differed. As no infected white-tailed deer were found in this outbreak until 2001 (Wobeser, 2009;

Shury and Bergeson, 2011), it is very unlikely that white-tailed deer were the source of this outbreak and that elk are the major reservoir species in this episystem. The monomorphic strain type found within all white-tailed deer sampled suggests they are more likely a spillover host in this episystem, due to the limited strain diversity found within this host species.

The spatial distribution of *M. bovis* in this ecosystem is also regionally confined, considering the long period of time during which elk have likely been infected. It is likely that elk have been dispersing from the area designated the core area within RMNP for quite some time, as this is considered to be some of the best elk habitat within this region. There is one documented case (Brook, 2007) of a dispersing bull elk which was collared in the core area within RMNP in February 2002 as a 3 year-old and was found to be infected with the same spoligotype and MIRU-VNTR type in an adjacent protected area 35 kilometres to the north of RMNP (Duck Mountain Provincial Park and Forest) in February 2005. As no other infected elk, white-tailed deer or cattle have ever been detected in the Duck Mountains, despite intensive repeated surveillance, it is very likely that this bull elk became infected within the core area of RMNP and later dispersed to the Duck Mountains, without infecting local cattle or wildlife. This elk likely represents but one example of many others which have dispersed this direction over time, and yet the infection has not become established in the Duck Mountains or in other adjacent areas. Two other bulls that dispersed to the same area over the same time period were found to be uninfected.

Access to round hay bales, left in fields in winter months, which are shared between wildlife and cattle, has been suggested as the major transmission mechanism for *M. bovis* in this episystem (Lees et al., 2003; Lees, 2004; Gooding and Brook, 2014). The findings of this research support these findings, as elk, and not white-tailed deer share closely related

miruspoligotypes in time and space. It is not possible to say exactly when transmission likely occurred based on these findings, but it is more likely that transmission would occur in winter months when mycobacteria survive longer in the environment, wild ungulates are typically in poor body condition and elk occur in larger herd sizes, which may facilitate transmission on a local scale. Also, infected elk have been present on the landscape for much longer, and with higher prevalence than deer since the first infected elk was killed by a hunter in October of 1992, and the first infected white-tailed deer was only discovered in 2001. Prevalence has also been much lower in elk than deer over time (Shury and Bergeson, 2011), suggesting deer are a spillover host in this episystem.

The two MLVA types found in this study are unique isolates in Canada, and have only been described from this limited geographic region in southwestern Manitoba. They have not been isolated from human or animal samples to date elsewhere in Canada, or elsewhere in the world (no spoligotypes or MIRU-VNTR match in mbovis.org database or SITVIT database). This indicates that there is very limited zoonotic transmission of this strain type within Canada, but the potential still exists, if these genotypes persist in domestic or wild populations. These typing data will be very valuable in future if this particular strain type does appear in human cases. Whole genome sequencing of mycobacteria within wildlife episystems is beginning to show previously unknown chains of transmission, and will likely be a promising further step to elucidate chains of transmission among individual elk and deer within this area in future.

In conclusion, very limited strain diversity exists within the episystem in southwestern Manitoba, with only 4 distinguishable strain types found using 24 loci MIRU-VNTR in combination with spoligotyping, consisting of two spoligotypes and two MIRU-VNTR types. One spoligotype was restricted to five cattle herds identified in 1990 and 1991, and was not

found in wildlife species. White-tailed deer *M. bovis* isolates had a monomorphic strain type restricted to a single spoligotype and MLVA type, but also had the lowest number of isolates of all three species. Elk isolates included two strain types, one of which exhibited moderate spatial clustering. These findings corroborate previous research which postulated the status of white-tailed deer as a spillover host in this episystem, and elk as the primary wildlife reservoir. There was significant spatial overlap of wildlife and cattle *M. bovis* isolates, which was used to delineate a 1366 sq. km core area in the western side of Riding Mountain National Park where management activities are now focused, and where spillover and spillback of *M. bovis* has occurred since at least 1992. The relative simplicity of this episystem compared to other wildlife reservoirs of *M. bovis* has allowed significant progress on control and management of the disease to be achieved in the past decade, despite being located within a national park.

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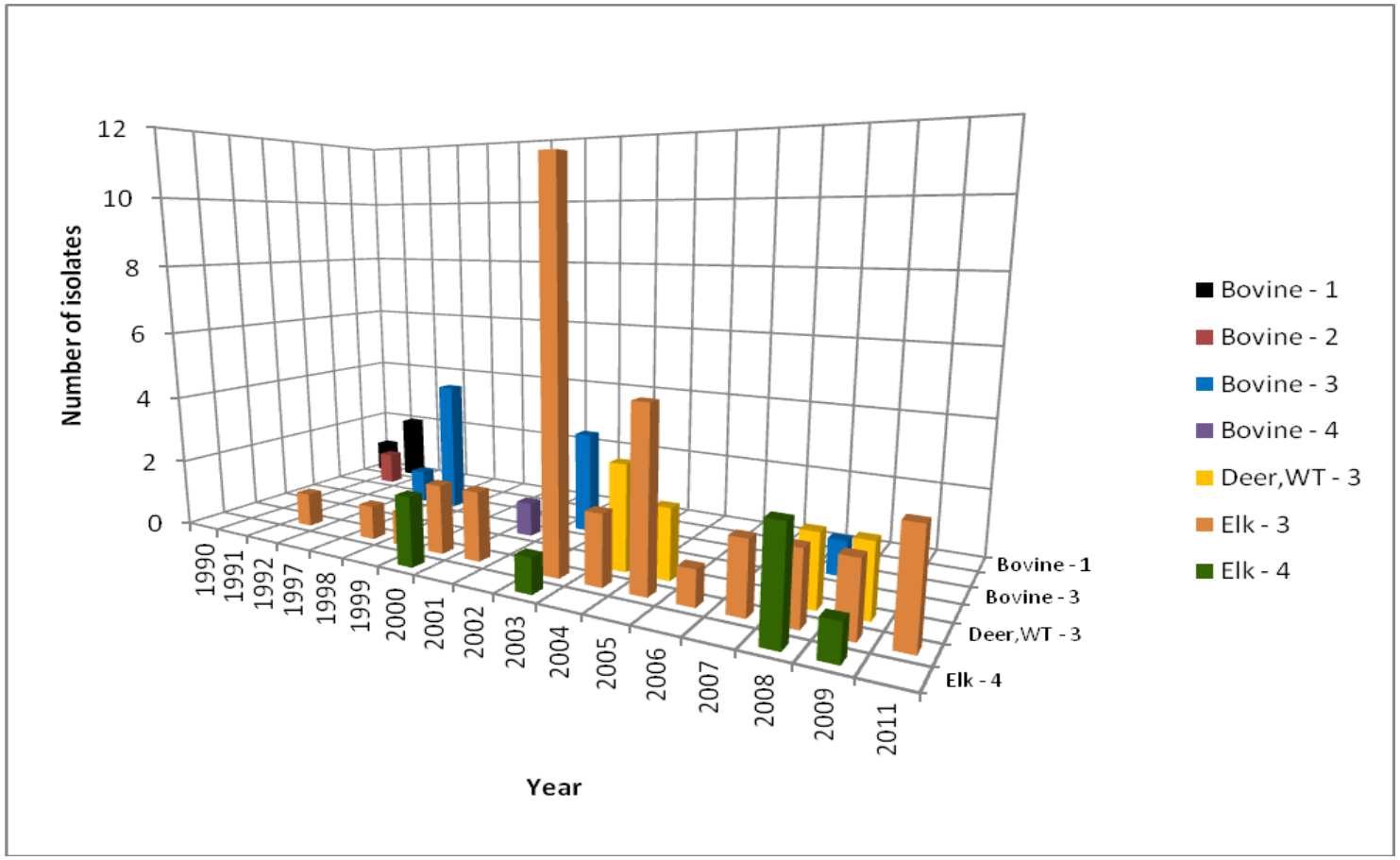

Figure 5.1 Miruspoligotypes isolated by host species, miruspoligotype and year from 1990 to 2011 in the GRME.

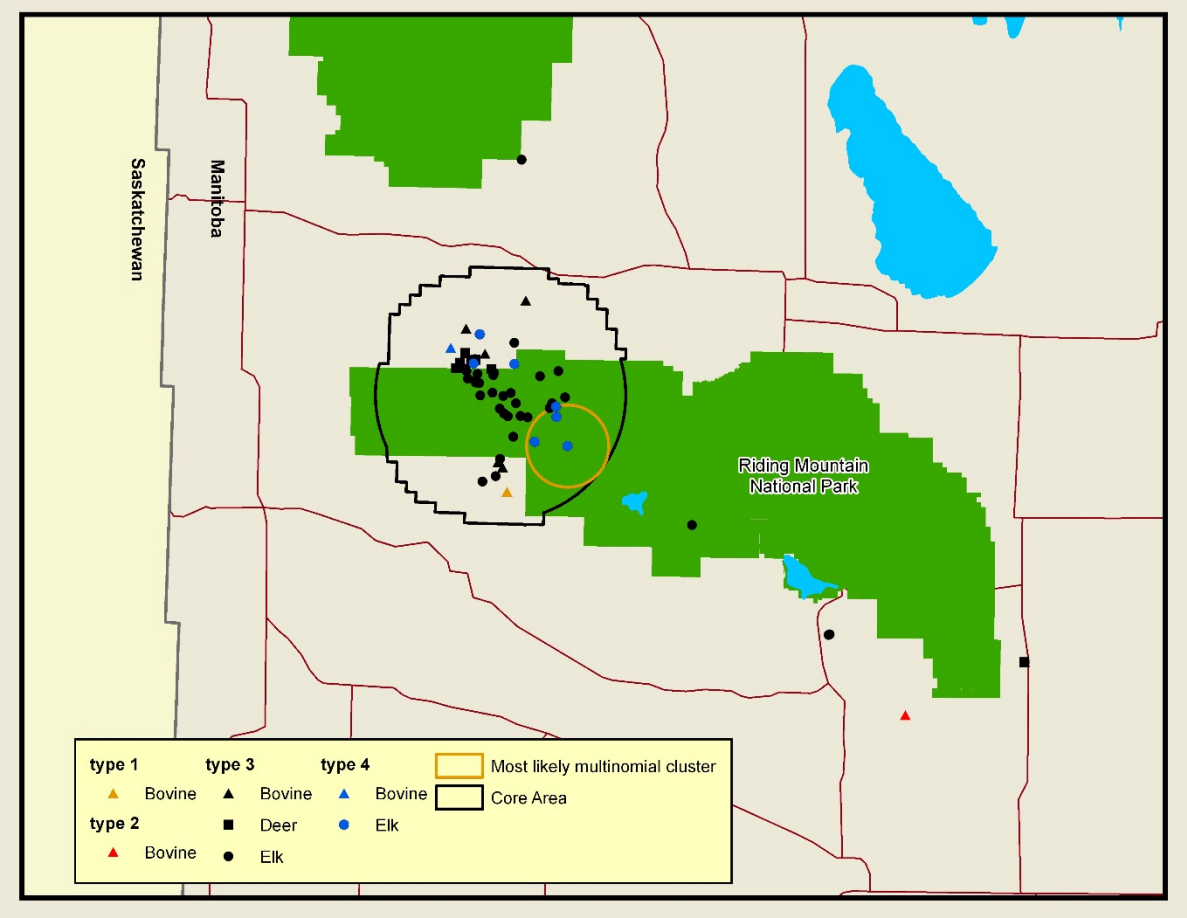

Figure 5.2 Spatial distribution of miruspoligotypes by host with most likely cluster from multinomial analysis and designated Core Area from binomial analysis.

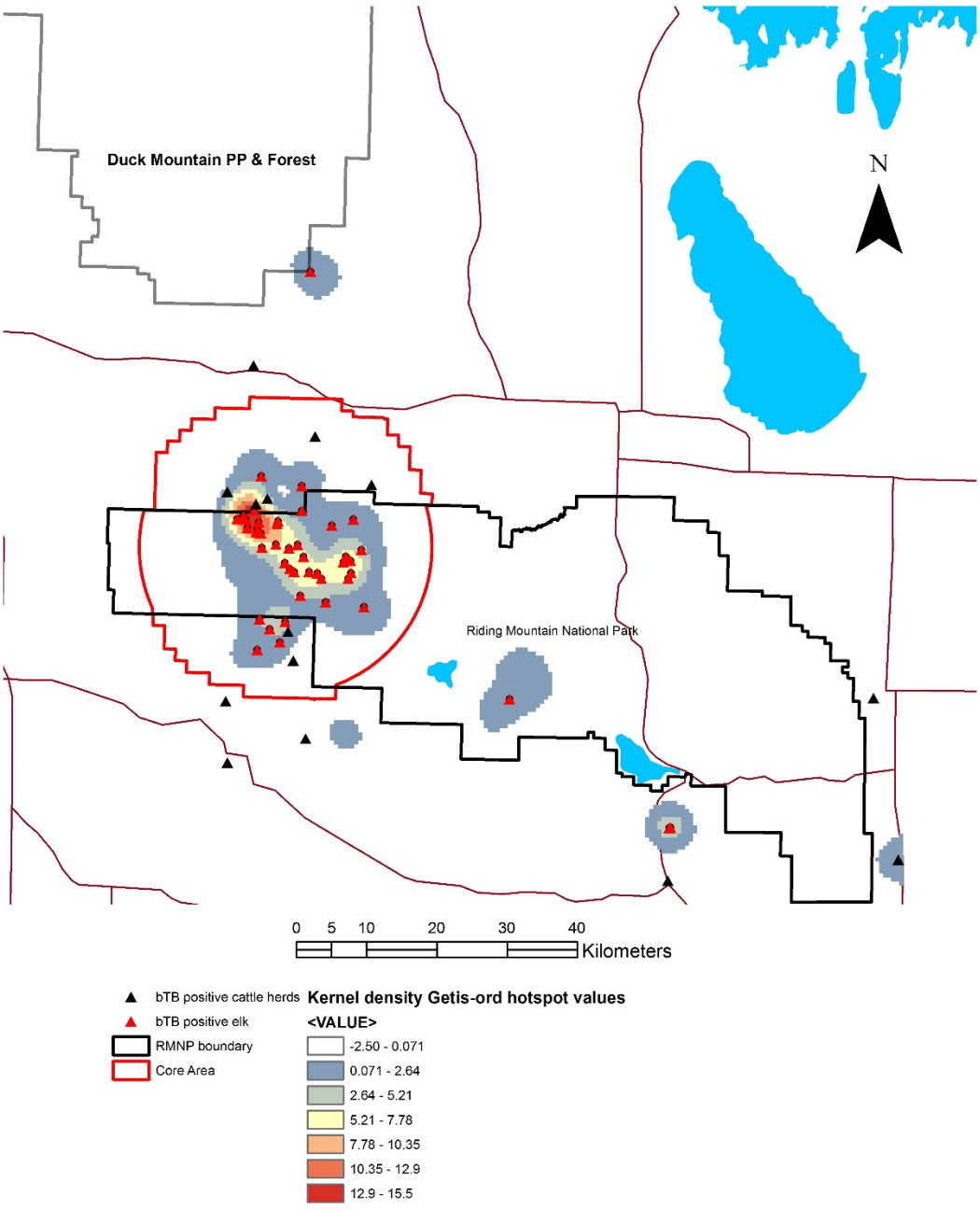

Figure 5.3 Getis-Ord hotspot analysis for bTB positive elk and white-tailed deer in the GRME.

Table 5.1 Frequency of miruspoligotype, host species and miru loci copy numbers for *M. bovis* isolates from 1990 - 2011 in the GRME.

		MIRU loci (alias)																																
		154 (MIRU 02)	424 (Mtub 04)	577 (ETR-C)	580 (MIRU 04)	802 (MIRU 40)	960 (MIRU 10)	1644 (MIRU 16)	1955 (Mtub 21)	2059 (MIRU 20)	2163b (QUB 11b)	2165 (ETR-A)	2347 (Mtub 29)	2401 (Mtub 30)	2461 (ETR-B)	2531 (MIRU 23)	2687 (MIRU 24)	2966 (MIRU 26)	3007 (MIRU 27)	3171 (Mtub 34)	3192 (MIRU 31)	3690 (Mtub 39)	4052 (QUB 26)	4156 (QUB 4156)	4348 (MIRU 39)	Bovine (%)		Elk (%)		WT deer (%)		All hosts combined (%)	
Miruspoligotype	SB code																																	
1	SB1070[a]	2	2	5	2	2	2	2	3	2	4	9	3	4	5	4	2	6	3	3	3	2	4	1	2	3	(17.6)	0	(0.0)	0	(0.0)	3	(4.4)
2	SB1070	2	2	5	2	2	2	2	3	2	4	8	3	4	5	4	2	6	3	3	3	2	4	1	2	1	(5.9)	0	(0.0)	0	(0.0)	1	(1.5)
3	SB1071[b]	2	2	5	2	2	2	2	3	2	4	9	3	4	5	4	2	6	3	3	3	2	4	1	2	12	(70.6)	35	(83.3)	9	(100.0)	56	(82.4)
4	SB1071	2	2	5	2	2	2	2	3	2	4	8	3	4	5	4	2	6	3	3	3	2	4	1	2	1	(5.9)	7	(16.7)	0	(0.0)	8	(11.8)
																										17		42		9		68	

[a] – previously designated MB-2 by Lutze-Wallace et al 2005

[b]- previously designated MB-1 by Lutze-Wallace et a2005

CHAPTER 6: OUTPUT BASED STANDARDS FOR DETERMINING FREEDOM FROM *MYCOBACTERIUM BOVIS* IN A WILDLIFE RESERVOIR IN SOUTHERN MANITOBA

While the previous chapters primarily dealt with retrospective analyses, this chapter attempts to look to the future, so that a scientifically sound basis for surveillance in wildlife populations can be justified. Ongoing wildlife surveillance is enormously expensive and all wildlife management agencies are interested in defining a potential 'stopping point', when further surveillance for M. bovis will be unnecessary and potentially discontinued. When this point will occur is open to speculation, especially for a disease with very low prevalence. This chapter provides a model for determining when the ecosystem could be considered free of M. bovis infection in wild cervids and how that might potentially be accomplished, using various surveillance strategies.

6.1 Introduction

Proving freedom from disease in populations is challenging in wildlife due to enormous costs involved with enumeration of population at risk, difficulties getting representative samples and lack of validated diagnostic tests for many diseases (Wobeser, 2006). Estimates of disease freedom typically rely on representative surveys of populations to estimate probability that a particular disease, if present, would be detected at a certain level (design prevalence) (Cameron and Baldock, 1998). Unfortunately, most wildlife surveillance data is neither representative nor does it come from a single source, such as a representative survey, but from multiple sources that are often biased. Recently, there has been interest in developing techniques to estimate freedom from disease in domestic animal populations using output based standards which can also utilize surveillance data from multiple sources, making them ideal for use in wildlife disease surveillance (Martin et al., 2007a; 2007b; Frossling et al., 2013). Most studies have used these techniques in domestic populations, where surveillance data already exists or is easily obtained, but there a few select projects that have used output based standards to estimate freedom from disease in wild populations (Wahlstrom et al., 2011).

Canada has been involved in a long term program of elimination of bovine tuberculosis from the national cattle herd since 1923 (Harrington et al., 2014). By 1985, Canada's domestic cattle

herds were considered officially tuberculosis free (OTF) by the World Organization for Animal Health (OIE) and the Health of Animals Act. Sporadic cases of bovine tuberculosis have occurred in the 1990's and have occurred in some provinces as late as recently as 2011 (Harrington et al., 2014). In addition to these sporadic occurrences, there have also been two well characterized wildlife reservoirs associated with wood bison in northern Alberta and the Northwest Territories and in southern Manitoba, associated with elk in and around Riding Mountain National Park (Nishi et al., 2006; Wobeser, 2009).

An area in southern Manitoba surrounding Riding Mountain National Park which includes the Duck Mountain Provincial Forest, designated the Greater Riding Mountain Ecosystem (GRME), contains elk and white-tailed deer infected with *Mycobacterium bovis* with subsequent spillover into domestic cattle. A subset of this area comprising Game Hunting Areas 23 and 23A and designated the Riding Mountain Eradication Area (RMEA), surrounding Riding Mountain National Park (Figure 1.1), was enacted in January of 2003 to allow zoning and restrictions of cattle movement until ante mortem whole herd testing could be completed. Management actions, including whole herd testing of cattle, initiated in 2001 resulted in the RMEA being considered free of infection with *M. bovis* in cattle in 2006, in accordance with the Health of Animals Act. Management actions have been highly focused since 2010 on a designated core area, within which elk and deer are 39.8 times more likely to be infected with *M. bovis* than the same species in areas outside, and within which 90% of infected elk and deer and 90% of infected cattle herds are located. Prevalence of *M. bovis* in elk and deer within the RMEA is approaching the lower limits of detection using combinations of hunter harvest, selective culling and opportunistic surveillance which has been in effect since 1998. Surveillance results from wildlife sampling in

2011/12 and 2012/13 did not detect any *M. bovis* culture positive results from a sample of 1,761 elk and white-tailed deer.

Wildlife reservoirs of *Mycobacterium bovis* are particularly challenging to detect or assess due to the chronic nature of the disease, potential latency, considerable spectrum in pathological changes, and challenges with diagnostic testing in wild species (Palmer, 2013). The objective of this chapter was to develop a scenario tree model for proving freedom from infection with *M. bovis* in elk and white-tailed deer in the RMEA. This model will then be used to estimate probability of freedom in elk and deer in the RMEA as well as surveillance system sensitivities for different components. Various options and future scenarios will then be explored to develop surveillance strategies and policies for wildlife testing in the RMEA in the coming decade, if subsequent testing finds that elk and deer populations test negative.

6.2 Methods

A stochastic scenario tree model was developed using the methodology of Martin et al (2007a; 2007b) and cumulative surveillance data collected annually from free-ranging elk and deer in the RMEA since 1997. *Mycobacterium bovis* is a chronic infection in elk and white-tailed deer, so annual time steps were considered appropriate for this model. These models are developed to determine the probability that a target population is free from disease, at a target or design prevalence, given the surveillance that has been undertaken to date. They are stochastic, in that many inputs are in the form of prior distributions based on available data or expert opinion in some cases. In the case of the RMEA, structured wildlife surveillance began in 1997 in response to a finding *M. bovis* in free-ranging elk near the park in 1992 and the finding of two infected nearby cattle herds in 1997 (Lees et al., 2003; Lees, 2004), and has continued annually to present. Three main surveillance components were incorporated into the model consisting of

elk and white-tailed deer sampled through; 1) hunter harvest, 2) selective culling (blood sampling), and 3) opportunistic sampling (Figure 6.1).

6.2.1 Surveillance data

Surveillance data were collected from long-term structured surveillance for *M. bovis* in wild cervids from the area in and around Riding Mountain National Park (RMNP) conducted jointly by Manitoba Conservation and the Parks Canada Agency (Shury and Bergeson, 2011; Shury et al., 2013). Surveillance consisted of samples collected from sport hunters through licensed hunting activities (hunt component), samples collected from wildlife found dead, which included primarily road-killed animals and predator-killed animals (opportunistic component), animals culled for density reduction programs (non-selective culling), and animals that were blood tested with removal of positives or suspects (selective culling). White-tailed deer and elk were the only two species included in surveillance samples; moose were excluded, as no *M. bovis* culture positive moose have been found since surveillance began, despite having sampled approximately 630 moose between 1997 and 2013 (all of which were culture negative for *M. bovis*). Data for model inputs were collected over a 4 year period (fall of 2010 to spring of 2014), during which it was believed that no further positive elk or deer would be detected, however, one positive cow elk was detected in the final year of surveillance.

Hunter sampled animals were collected through an active communications program that required hunters to submit a complete head and lung pluck from harvested animals. Specimens were collected through the months of August to February every year from 1997 to 2013. A complete set of head and lungs was not always obtained and many samples were either too autolyzed or damaged by gunshot trauma to be useful. Opportunistic samples were collected when found through public reporting or through other surveillance and research programs (radio-telemetry). Non-selective culling occurred sporadically throughout the study period with an area-

wide surveillance cull of 226 white-tailed deer in March of 2004 using ground-based agency sharpshooting in the entire RMEA. Non-selective culling for both elk and white-tailed deer occurred within a designated Core Area (Figure 4.1) during the winters of 2008/2009, 2009/2010 and 2013/2014 with the primary goal of reducing elk density, as there was evidence that cervid density contributes to transmission and maintenance of *M. bovis* in cervid populations (O'Brien et al., 2006; Vicente et al., 2007a). Both selective and non-selective culling of white-tailed deer was done to both reduce density and determine where infected deer were present, as most samples were obtained through hunting originated from outside Riding Mountain National Park, and very few samples were obtained through opportunistic sampling.

Blood testing (selective culling) for *M. bovis* in elk began in the winter of 2000/2001and was continued annually until 2013/2014, while blood testing for white-tailed deer began in the winter of 2004/2005 continuing annually until 2013/14. Animals were captured using helicopter net gunning during winter and late spring (December through May) using methods described in detail in Shury et al. (2013). Briefly, adult cow elk were initially captured in January or February, while adult bull elk were captured in April after antlers were dropped. A two-stage sampling strategy was employed for the blood testing surveillance component. Three different blood based assays were used as initial screening tests interpreted in parallel; a fluorescence polarization assay (FPA), a lymphocyte stimulation test (LST) and a lateral flow chromatographic assay (Cervid TB Stat-Pak). Animals that were positive on any 1 of these 3 tests were recaptured from 1 to 60 days later and euthanized with a captive bolt gun and subjected to a complete necropsy examination and culture for *M. bovis*. Animals that were negative on all three screening tests were not recaptured, except by chance in subsequent capture years through the blood testing program.

6.2.2 Model Inputs

The scenario tree model was constructed in Microsoft Excel 2007 (Microsoft Corporation) and analyzed using @Risk for Excel version 6.2.0 (Palisades Corporation Inc.). The model only includes an animal level infection node and does not include higher levels of clustering which in domestic animal surveillance would include a herd level infection node. The concept of 'herd' is difficult to apply to free-ranging cervid populations as these animals do not respect jurisdictional or property boundaries and are essentially free to move wherever they wish. These movements vary seasonally and vary by sex, because of difficulties estimating herd level inputs, it was decided to restrict the model to a single animal level infection node for reasons of simplicity.

Three levels of risk are present in the model, represented by risk nodes for: 1) geographic zone where animal was sampled, 2) species of animal (elk or white-tailed deer), and 3) sex of animal. Age was considered as a potential risk factor, but was rejected due to difficulty estimating the age structure of populations on an annual basis. Geographic zones were determined using a spatial analysis of elk and deer sampled from 1997 to 2011 using the spatial scan statistic (Chapter 5). The RMEA was split into three separate zones with varying degrees of risk; the core area, RMEA west and RMEA east (Figure 6.2). The core area was a circular zone which was the most likely cluster found using a Bernoulli purely spatial analysis with the spatial scan statistic. The RMEA west was the area outside the core area west of Highway 10, while the RMEA east is the remaining area east of Highway 10, which is a convenient geographic feature that separates elk sub-populations in the west and east of the park (Vander Wal et al., 2013a). There is little, if any genetic interchange between these two sub-populations and no radio-collared elk have been detected moving from areas west of Highway 10 to areas east of Highway 10 or vice versa for the past 15 years (RMNP unpublished data). The relative risk of a wild elk or deer being culture positive in the core area was 39.8 times compared to animals outside this zone

within the RMEA. This risk value was modeled as a beta pert distribution with a minimum value of 5.9 (lower 95% confidence interval from logistic regression model for core area), a most likely value of 16.2 (mean value from restricted logistic regression value) and a maximum value of 44.3 (upper 95% confidence interval from restricted logistic regression model). An intermediate level of risk was ascribed to the RMEA East with a minimum value of 1, a most likely value of 2 and a maximum value of 3, based on the fact that culture positive elk were found in this region historically, but not recently, with the last culture positive elk being found in 2000. One culture positive elk was also detected in the RMEA west, in 1999 with no positive detections since that time. This zone was used as the reference zone with a risk value of 1. Risk values for species of animal sampled were based on the prevalence ratio for sampling completed between 1997 and 2009. The annual apparent prevalence from all methods of surveillance were pooled annually (only 5 years included data for both positive elk and deer 2003/2004, 2004/2005, 2007/2008, 2008/2009, and 2009/2010). The resulting prevalence ratio range was used as the basis for a beta pert distribution, with a minimum value of 2.12, a most likely value of 6.65 (mean value for all years), and a maximum value of 16.2. Risk values for sex were also derived from the restricted logistic regression model (Chapter 4) and modelled as a beta pert distribution using lower 95% confidence interval for the minimum value, mean for the most likely value and upper 95% confidence interval for the maximum value (

The hunt and opportunistic limbs of the tree contained identical limb detection probabilities which consisted of two detection nodes; 1) the probability of detecting gross visible lesions (GVL) at post-mortem inspection, and 2) the probability of detecting *M. bovis* by culture in animals with GVL. Animals without evidence of GVL at post-mortem inspection were considered negative, while animals with negative culture for *M. bovis* following culture of

tissues with GVL were also considered negative. Animals with both GVL and with a subsequent positive culture for *M. bovis* were considered positive. Typically hunters were asked to submit both head and lungs from animals they killed, but only one body part was often available or considered useful for surveillance. Sensitivity of detection for GVL was assigned based on the probability of a GVL being present in the body part available in animals where the whole body was examined. For example, there were 32 out of 38 elk and deer examined with positive *M. bovis* cultures that had a GVL in either the head or lung at necropsy for a sensitivity of 84%. These sensitivities were modeled as beta distributions based on values applied using Betabuster software.

The blood test limb of the tree included both the GVL and culture detection nodes, but animals submitted through this surveillance component had an additional detection node as they were pre-screened prior to necropsy using three different blood-based assays which were interpreted in parallel during an initial capture episode. Both elk and deer were captured by helicopter net-gun during winter months and blood was collected by jugular venipuncture as described in Shury & Bergeson (2011). Radio-collars were applied and animals subsequently testing positive to a lymphocyte stimulation test (LST), fluorescence polarization assay (FPA), or Cervid TB Stat-Pak were recaptured and subjected to a complete necropsy as described in Shury et al (2014). Animals that tested negative to all three tests were considered negative for *M. bovis* as the negative predictive value of this serial testing is 100%. Sensitivity of blood testing was modeled as a beta pert distribution with a minimum value of 0.86 (lower 95% confidence interval for sensitivity estimate from Shury et al 2014), a most likely value of 0.99 (author expert opinion), and a maximum value of 1.0.

Two categorical nodes were included the model to reflect the distribution of wildlife within the RMEA and the different surveillance components included in the model. After accounting for zone of sampling (core, RMEA west, RMEA east), the RMEA was further divided into 3 distinct subzones (Figure 6.2) based on whether animals were sampled inside or outside of Riding Mountain National Park (RMNP). This was done to reflect the very different population proportions of elk and deer which occur inside and outside RMNP and the different sampling strategies that were employed in each subzone. Hunting was the primary surveillance component in the RMEA outside RMNP, while blood testing or direct removal were the primary surveillance strategies employed within RMNP. For modeling purposes, animals removed by helicopter net-gun capture and euthanasia by captive bolt without prior blood testing (non-selective culling) are considered equivalent to hunter killed animals with the exception that the whole body was examined, rather than just the head and lungs.

The design prevalence is the level of disease that the surveillance system can potentially detect, in this case *M. bovis* in elk and deer within the RMEA. The animal level design prevalence for the model was assumed to be 0.001 (0.01%). This level is consistent with international standards for determining freedom from bovine tuberculosis in domestic animals (World Organization for Animal Health, 2014). It is also approximately consistent with one infected wild cervid in one of the 9 subzones at a prevalence of 1% (1/9 X 0.01 = 0.0011), which is a design prevalence recommended by the Scientific Review Committee of the Manitoba Bovine TB Task Force (pers. comm. P. Paquet). This is quite a conservative value for a wildlife population (Tataryn, 2009), and is even more conservative than the guideline offered by some authors which is that a value of 10% of the lowest prevalence estimate be used for wildlife species with little or no data (Wahlstrom et al., 2011). For the RMEA, the lowest annual apparent

prevalence has been 0.07 (0.7%) found in 2005/2006 (Chapter 4), resulting in an estimated design prevalence of 0.007 (10% of 0.07).

6.2.3 Surveillance sensitivity calculations

Unit sensitivities (probability that if an animal is infected, the diagnostic tests being used will detect it) were calculated by multiplying the detection probabilities used for each limb of the tree (hunt, opportunistic, blood test). For the blood test limb, this was the sensitivity of the three blood tests in parallel multiplied by the sensitivity of culture and necropsy as described above. For the hunt and opportunistic trees, these were the product of the sensitivity of the body parts examined by the sensitivity of culture and necropsy.

Calculation of risk for each combination of risk levels was incorporated into the model by calculation of an adjusted risk for each stratum formed by the three different risk nodes (zone, species, sex) according to the methodology described by Martin et al (2007b) to ensure that the weighted average risk for each stratum was equivalent to 1. For two different risk categories (high risk [H]and low risk [L]), adjusted risk was calculated as;

$$Adjusted\ Risk_{Core} = \frac{RR_{Core}}{(PPr_{Core}\ X\ RR_{Core} + PPr_{RMEA\ West} X\ RR_{RMEA\ West} + PPr_{RMEA\ East}\ X\ RR_{RMEA\ East})} \ldots.(6.1)$$

Where PPr_{Core} is the population proportion of animals in the core area, RR_{Core} is the relative risk for the core area, $PPr_{RMEA\ East}$ is the population proportion of animals in the RMEA East and $RR_{RMEA\ East}$ is the relative risk for the RMEA East

Adjusted risk for species was calculated in a similar fashion using the following equation:

$$Adjusted\ Risk_{Elk} = \frac{RR_{Elk}}{(PPr_{Elk}\ X\ RR_{Elk} + PPr_{WTD} X\ RR_{WTD})} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(6.2)$$

Where RR_{Elk} is the relative risk value for elk from the restricted logistic regression model, PPr_{Elk} is the population proportion composed of elk, RR_{WTD} is the relative risk value for white-

tailed deer from the restricted logistic regression model, and PPr_{WTD} is the population proportion composed of white-tailed deer.

And adjusted risk for sex was calculated using the following equation:

$$Adjusted\ Risk_{Male} = \frac{RR_{Male}}{(PPr_{Male}\ X\ RR_{Male} + PPr_{Female} X\ RR_{Female})} \quad \text{.....(6.3)}$$

Where RR_{Male} is the relative risk value for male animals from the restricted logistic regression model, PPr_{Male} is the population proportion composed of males, RR_{Female} is the relative risk value for female animals from the restricted logistic regression model, and PPr_{Female} is the population proportion composed of females.

The product of these adjusted risks with the design prevalence were used to calculate an effective probability of an animal being infected (EPI) for each stratum formed by each of the three risk groups in combination (n = 36) using the following formula;

$$EPI_k = P_A^*\ X\ AR_k^{Species}\ X\ AR_k^{Zone}\ AR_k^{Sex} \quad \text{.....(6.4)}$$

Where P*A is the design prevalence, $AR_k^{Species}$ is the adjusted risk value for eache species (k=2), AR_k^{Zone} is the adjusted risk value for each zone (k=3), and AR_k^{Sex} is the adjusted risk value for each sex (k=2).

Total surveillance system sensitivity was calculated by determining the probability that all sampled animals tested negative (PNegkA)

6.2.4 Modelled Scenarios

Nine separate potential future scenarios were modelled with the following parameters:

Scenario 1 - Hunting plus opportunistic only post 2013/14 using 2014/15 estimates (bucks only, no female harvest)

Scenario 2 – One Year Blood test (similar to 2013/14) plus hunting only post 2013/14 using 2014/15 harvest (no bucks)

Scenario 3 - Two Years Blood testing (similar to 2013/14) plus hunting only post 2015/16 using 2014/15 harvest (no bucks)

Scenario 4 - Single 100% core female blood test followed by hunter surveillance only post 2014 15

Scenario 5 - 80% core female blood test plus 20% following year followed by hunter surveillance only post 2014 15

Scenario 6 - 80% core female blood test plus 20% following year followed by hunter surveillance only post 2014 15 plus another similar round 2019/20 2020/21

Scenario 7 - Single 100% core female blood test followed by 50% reduction in hunting only post 2013/14 (50% of 2013/14 levels)

Scenario 8 - 80% core female blood test plus 20% following year followed by hunter surveillance only post 2014 15 at 50% harvest levels

Scenario 9 - 80% core female blood test + 20% following year followed by 50% hunter surveillance only post 2014 15 plus another similar round 2019/20 2020/21 with 50% hunter surveillance

Scenario 10 - Hunting plus opportunistic only post 2013/14 using 2014/15 estimates, with 20 additional elk harvested in Core North (5 bulls, 5 cows) and Core South (5 bulls, 5 cows)

6.3 Results

The model was very sensitive to the assumed design prevalence (P^*_A) and the probability of introduction (Pintro) (Figures 6.3, 6.4), both which had relatively large impacts on the overall system sensitivity (varied from 0.05 to 0.9 depending on design prevalence) and probability of freedom (mean varied from 78% to 95% in 20131/32 depending on Pintro value chosen). Other inputs such as deer and elk numbers had less influence on overall system sensitivity, dependent

in which zone animals were sampled and their gender and species (Figure 6.5), but still had a moderate impact. Of the three risk nodes, the most sensitive was Zone, followed by which species were sampled; the sex of the animal sampled was the least sensitive risk parameter (Figure 6.6). The population parameters for the SSC (Surveillance System Component) reference population (elk and deer in the entire RMEA) had relatively little effect on overall system sensitivity. Due to the method used for calculation of adjusted risk, which incorporates population proportions, elk from the Core North and Core South subzones had much higher effective probabilities of infection (EPI) than elk from within RMNP and thus, effectively were weighted higher in terms of surveillance value (Figure 6.7).

Probability of freedom and overall surveillance system sensitivities for the ten different potential future scenarios are presented in Figure 6.8. Scenarios 1,2 , 7, 8 and 9 failed to eventually reach a final mean probability of freedom of 95% by 2031/32, although scenarios 1 and 2 came very close by 2031/32. Other scenarios eventually reached a mean 95% probability of freedom at different time points with scenarios 6 and 10 reaching this threshold earliest in 2021/22.

During the 2011/12 and 2012/13 surveillance seasons (the only two seasons which had negative surveillance results), component sensitivity varied substantially depending on the year. Blood test component sensitivity was substantially lower than hunt component sensitivity in 2011/12, while in 2012/13, both components were very similar (Figure 6.9). Hunt component sensitivity dropped significantly in 2014/15 due to lower numbers of deer and elk being harvested. Opportunistic component sensitivities were substantially lower than the other two components in all years.

6.4 Discussion

Scenario tree modeling proved to be a valuable way to describe the wildlife surveillance system in the GRME as well as a useful future tool to demonstrate freedom from bTB in the GRME. Unlike most other input-based calculations that attempt to prove freedom from disease, scenario tree models allow the use of multiple surveillance system inputs and not just inputs from what are assumed to be randomized surveys (Martin et al., 2007b). *M. bovis* has been considered to be endemic among free-ranging elk and deer in the RMEA since first being discovered in 1992 (Lees et al., 2003; Wobeser, 2009), and recent work has demonstrated a significant decline in prevalence with eradication being likely in the near future (Shury and Bergeson, 2011; Shury et al., 2014). Two seasons of negative surveillance data (2011/12 and 2012/13) were followed by the finding of a single infected 10 year old cow elk from the core area of the RMEA in 2013/14. Due to very low elk densities that currently occur within the RMEA (Chapter 4) in combination with intensive domestic cattle surveillance since 2002 and on-farm risk mitigation measures including hay barrier fencing (Brook et al., 2013; Gooding and Brook, 2014), it is very likely that *M. bovis* has dropped to such low prevalence that it is unlikely to persist in these populations, without additional transmission from other reservoirs. This scenario tree model provides a very useful tool to be able to demonstrate freedom from *M. bovis* in elk and deer populations within the RMEA to help guide future domestic cattle testing and allow more targeted and effective surveillance strategies in the future.

The most sensitive model inputs were the assumed design prevalence (P^*_A) and the probability of introduction from year to year (Pintro), so careful consideration should be given to these parameters as they have the most influence on potential probabilities of freedom that are estimated. A design prevalence of 0.001 was chosen for current modeling scenarios as this is justifiable on three main grounds; 1) it is the animal level design prevalence recommended by

the OIE for freedom from bovine tuberculosis in farmed cervids (99.9% of farmed cervids in a zone free from bTB) as an international standard (Health, 2014), which is the first consideration in choosing this parameter (Martin et al., 2007b), 2) it is very similar to a design prevalence originally recommended by the SRC (0.0011), which was one infected subzone out of 9 (0.11) multiplied by an animal level design prevalence of 1% (0.01), and 3) it is quite a conservative value for a free-ranging species from which it is not logistically possible to capture and test every single animal in the population.

The probability of introduction is also a very difficult value to parameterize. It represents the probability that the agent (*M. bovis*) will be reintroduced to the target population between surveillance periods (years in this case) given that the population was free at the beginning of surveillance. I considered the current probability of introduction from domestic cattle spillover to be negligible, as both whole herd live animal testing and abattoir surveillance has been ongoing in the RMEA since 2000 and no positive cattle herds have been detected in the RMEA since May, 2008 , and the RMEA has been considered officially bTB-free according the Canada's Health of Animals Act and Regulations (CFIA, 2012). Reactivation of latent tuberculosis and transmission to other wild cervids is a potential route of reintroduction, but this is very difficult to parameterize, as latent *M. bovis* infection can occur in wild cervid populations, and there is debate about how significant and what proportion of animals remain latent or actually resolve infection (O'Reilly and Daborn, 1995; Nugent, 2005). Evidence from the RMEA suggests that an older cohort of female elk, born prior to 2004, remain infected, but that other age and sex classes of elk are not currently infected at levels detectable using current surveillance techniques. It is clear that this cohort is diminishing over time and that few *M. bovis* positive elk still reside in this cohort (Chapter 4). Any ongoing transmission would likely come from this cohort of

animals, which are aging every year and will likely die out in the next decade (Maximum recorded age of a female elk is 23 years), with a diminishing probability of introduction. Additionally, since current elk densities are at historical all-time lows for this population (Chapter 4), it is unlikely that further intra or inter species transmission will occur to allow *M. bovis* to persist in either elk or deer populations. For these reasons, the Pintro value of 0.01 is considered a conservative probability in this ecosystem.

Future surveillance for wildlife in the RMEA should likely focus on elk in the core area, as these animals are more heavily weighted in the scenario tree model and higher system sensitivity will be achieved by focusing on elk in this area. Maximum system sensitivities are achieved through focusing on the blood test component within the core area, but relatively high system sensitivities can also be realized through relatively large numbers of both elk and deer, depending on geographic zone, species and sex. Sensitivities are maximized if hunted samples are collected from the North and South Core areas, especially for elk. High priority should be given to these two particular areas if hunting is used as the major or only surveillance method in future.

Given current knowledge of the epidemiology of *M. bovis* infection in wildlife, it likely the earliest that a 95% confidence level of freedom from *M. bovis* would occur would be approximately 2022, based on current scenarios. This is entirely dependent on negative finding in all subsequent surveillance between present and 2022. If the assumption that intraspecific transmission among the major reservoir species, elk, substantially declined after 2004 due to concurrent management factors implemented at that time is correct, most of the elk cohort born after 2004 would be very aged (at least 18 years of age) and very low in number by 2022. The oldest animals in that cohort (born in 2005) are currently 9 years of age in 2014, leaving open the

possibility of latent *M. bovis* infection in a small proportion of these animals which could potentially transmit the infection later in life. Although this transmission is possible, it is also very unlikely if elk densities remain low (below 1 elk per sq. km) until at least 2022. Surveillance system sensitivity has dropped substantially in the past year (2014/15 – Figure 6.9) due to decreased numbers of hunter returns. This will likely pose a problem in future years if WTD and elk populations remain low. In this case, wildlife management agencies should consider conducting non-lethal surveillance (blood sampling with test and removal) as an alternative to hunting, to maintain annual surveillance system sensitivities at reasonably high levels and reach a 95% probability of freedom in the next decade.

Sporadic cases are likely to occur in this intervening span of time, but ongoing transmission will be unlikely and prevalence is likely to be so low as to be undetectable using current levels of surveillance. Sporadic cases of *M. bovis* infection have been recorded in wild cervids in North America while both US and Canadian cattle eradication programs were in their early stages, but these sporadic cases failed to become wildlife reservoirs (with the exception of Michigan), likely because of low cervid densities and also due to farming practices that kept wildlife and cattle separated. The only two exceptions, Michigan and the RMEA, both experienced high cervid densities and varying levels of artificial feeding and baiting, the two major factors that likely led to the creation of these wildlife reservoirs of *M. bovis*. Other reported cases of *M. bovis* infection in wild North American cervids seem to have involved spillover without creation of a reservoir. As a result, even though sporadic cases of *M. bovis* may occur within the RMEA in the next decade, these cases are likely to be extremely rare and unlikely to result in ongoing transmission, if the factors that created the wildlife reservoir no longer exist. During the next decade within the RMEA, it will be important to maintain consistent on-farm biosecurity to prevent cattle-elk/deer

contact, maintain low elk and deer densities and ensure enforcement of legislation banning baiting and feeding of wildlife.

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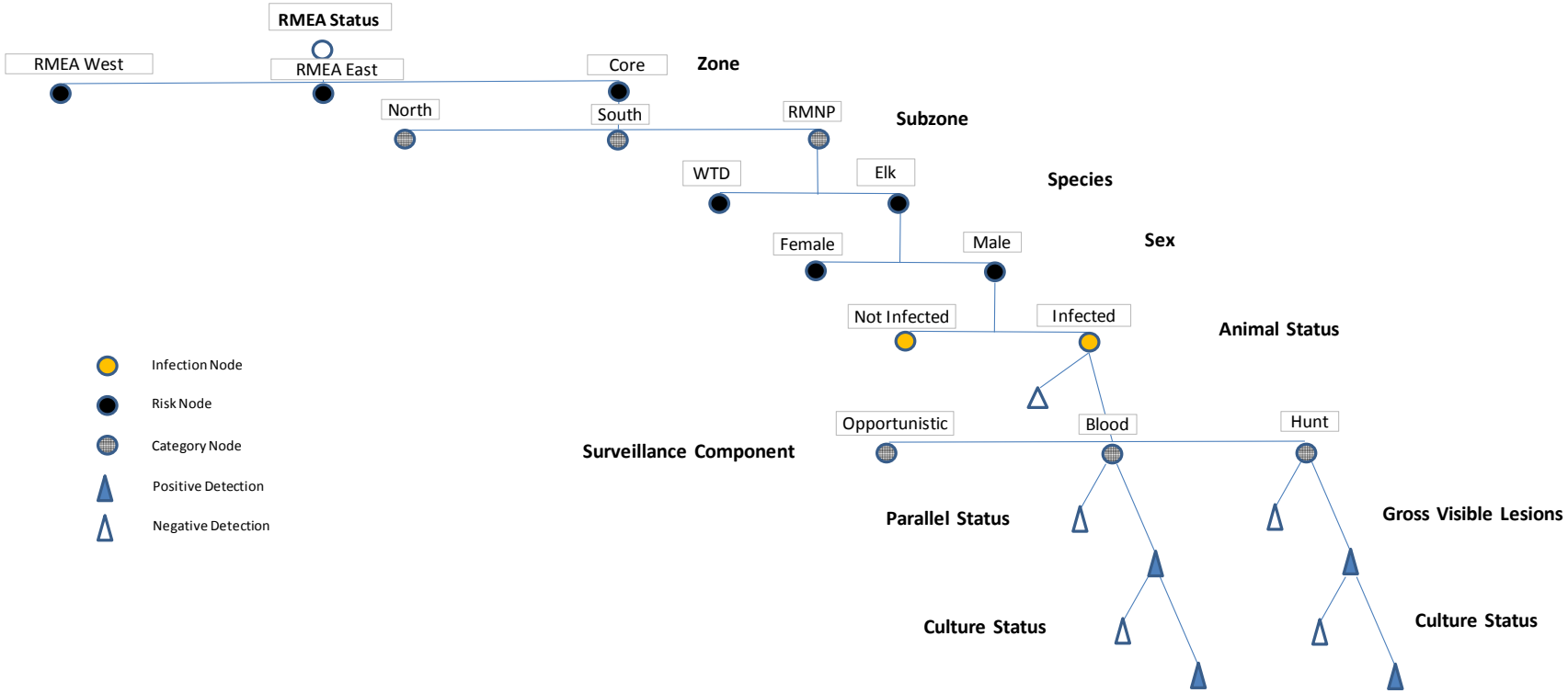

Figure 6.1 Scenario tree model structure for *M. bovis* in elk and white-tailed in the RMEA.

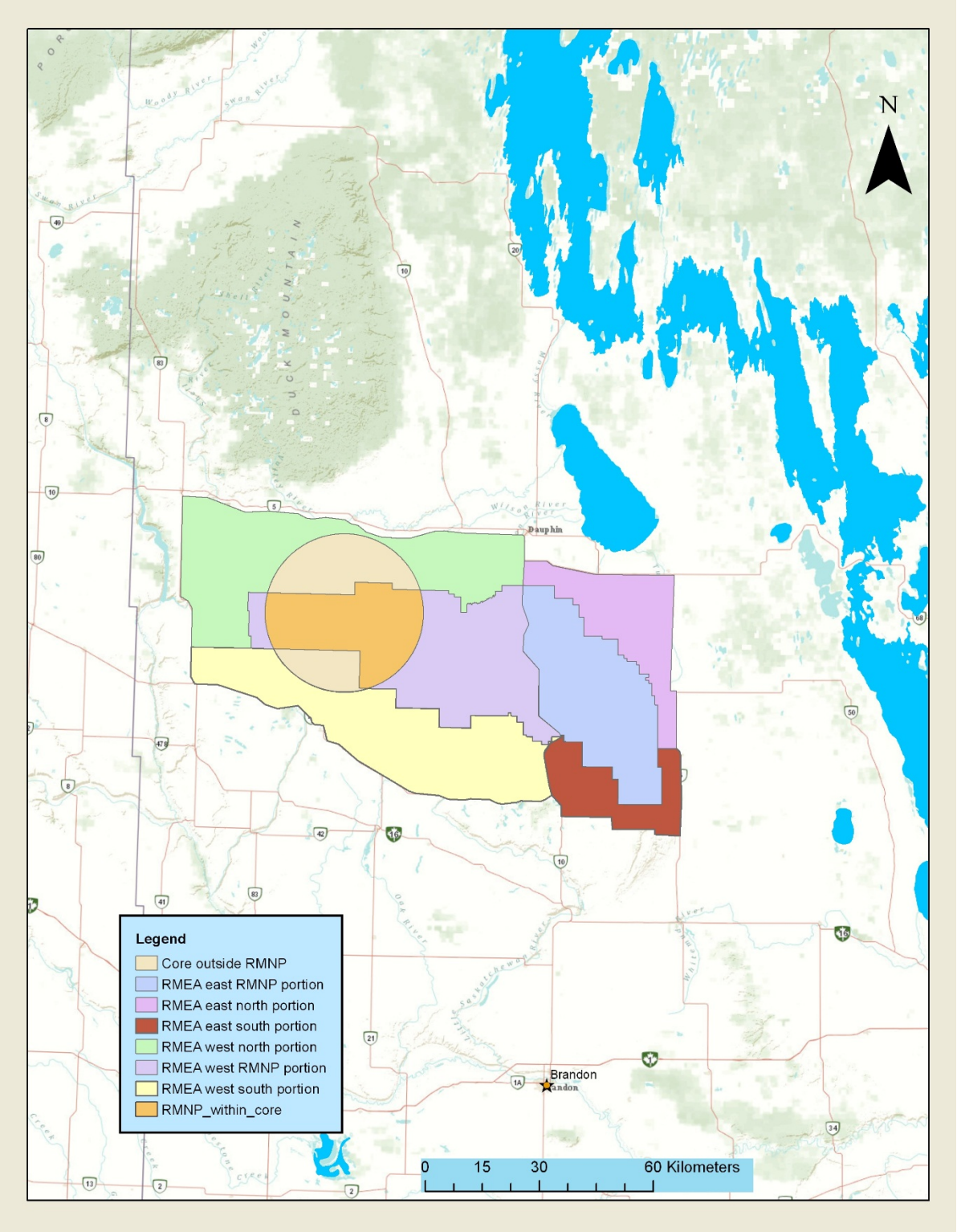

Figure 6.2 Zones and subzones of the RMEA used for scenario tree models

Table 6.1 Design prevalence and risk parameters for scenario tree model.

	Node	Surveillance Component	Input value	Source of information
Animal Level Risk				
	Species	Hunt , Blood, Opportunistic	β pert (2.12, 6.65, 16.2)	Prevalence ratio elk:WTD range
	Sex	Hunt , Blood, Opportunistic	β pert (1.22, 2.08, 3.53)	Restricted logistic regression
Zone Risk				
	Core Area	Hunt , Blood, Opportunistic	β pert (5.9, 16.2, 44.3)	Restricted logistic regression
	RMEA West	Hunt , Blood, Opportunistic	Ref	
	RMEA East	Hunt , Blood, Opportunistic	β pert (1, 2, 3)	Author expert opinion
P_star (P^*_A)		Hunt , Blood, Opportunistic	0.001	OIE guidelines, Scientific Review Committee
Sensitivity culture		Hunt , Blood, Opportunistic	β pert (0.67, 0.74, 0.79)	More et al 2009
Sensitivity Parallel Blood Test (FPA, LST, Stat-Pak)		Blood	β pert (0.86, 0.99, 1.0)	Shury et al 2014 plus expert opinion
Sensitivity GVL				
	Whole Body	Hunt & Opportunistic	β pert (0.9, 0.95, 1.0)	Author expert opinion
	Head & Lungs	Hunt & Opportunistic	β dist (21.9, 5.7)	Empirical data this study
	Head Only	Hunt & Opportunistic	β dist (14.9, 10.1)	Empirical data this study
	Lungs Only	Hunt & Opportunistic	β dist (11.0, 12.2)	Empirical data this study

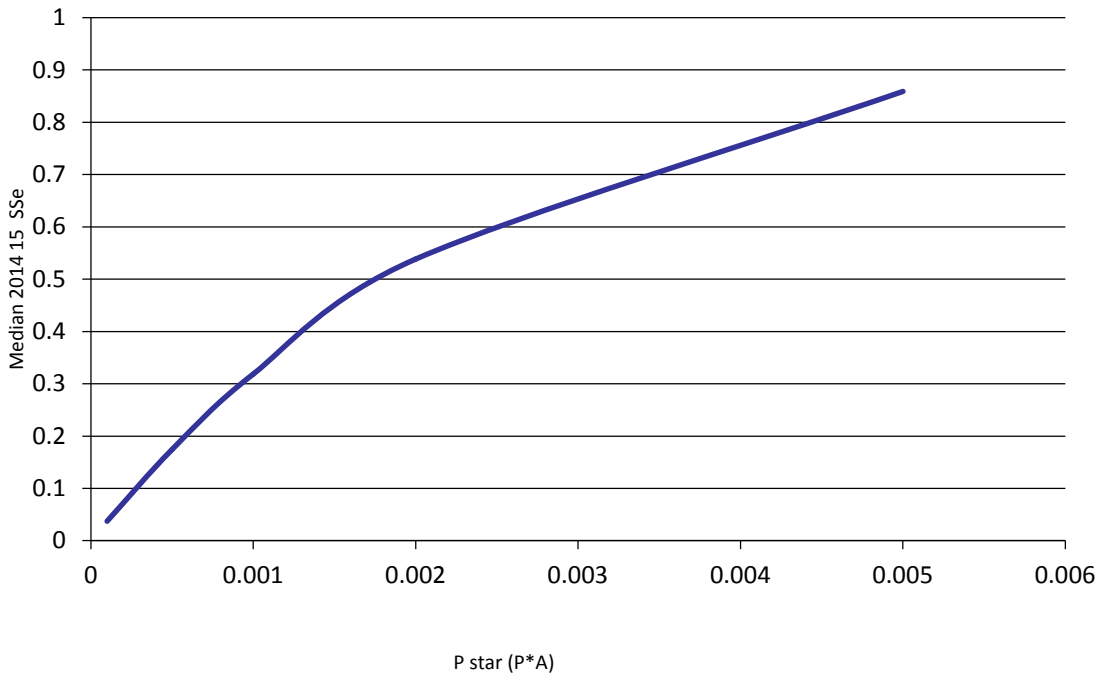

Figure 6.3 Sensitivity analysis: median change in 2014 15 system sensitivity (SSe) for various design prevalence values (P^*_A).

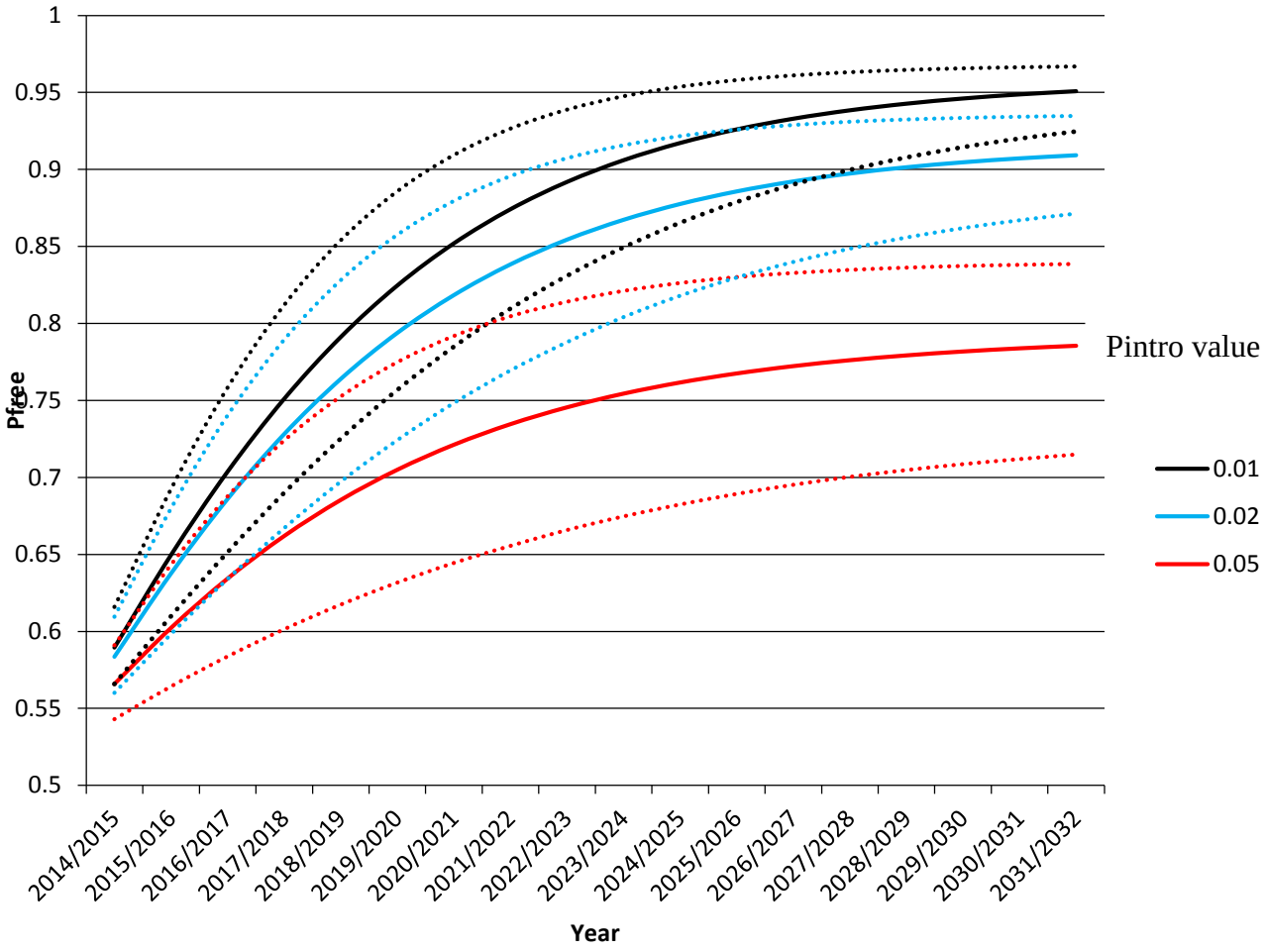

Figure 6.4 Probability of freedom (Pfree) with 95% confidence intervals for three different probability of introduction (P intro) values using Scenario 5.

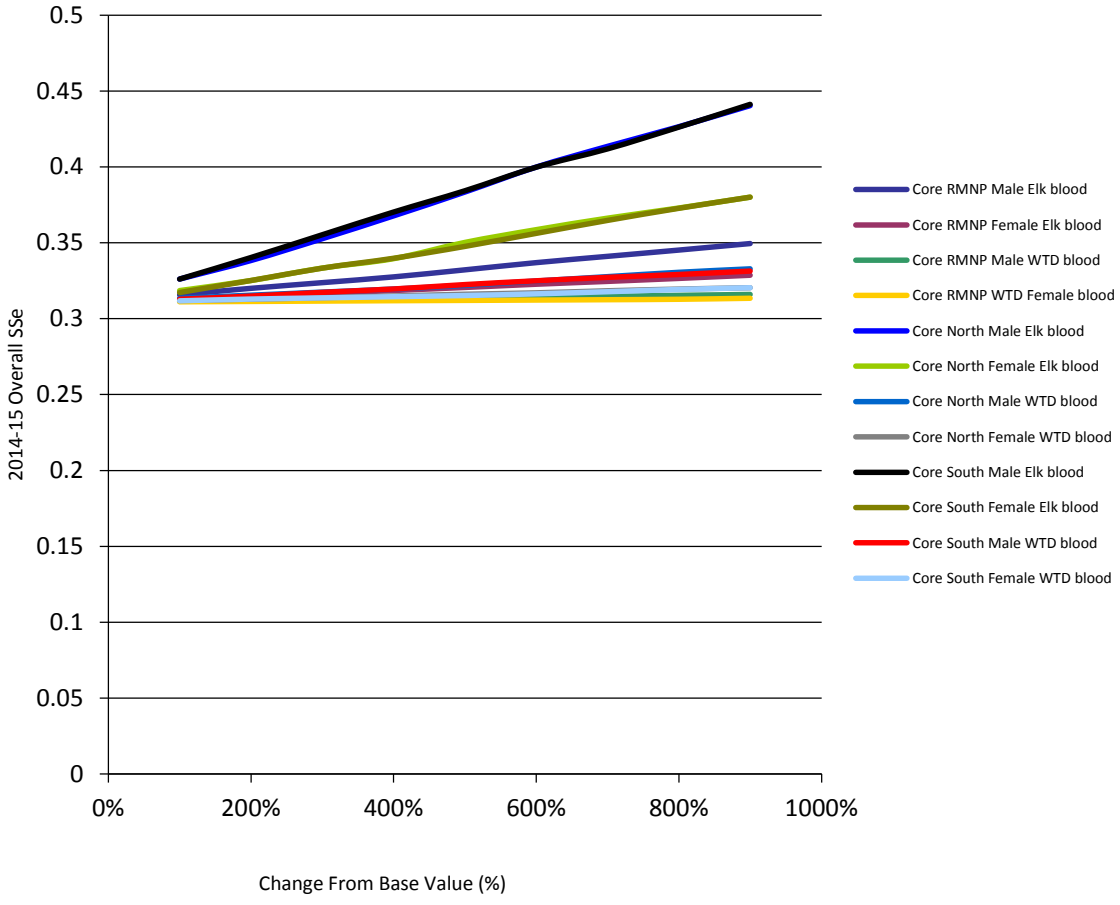

Figure 6.5 Change in overall system sensitivity (SSe) for range of input values from -100% to 100% change in 2014/15 surveillance numbers.

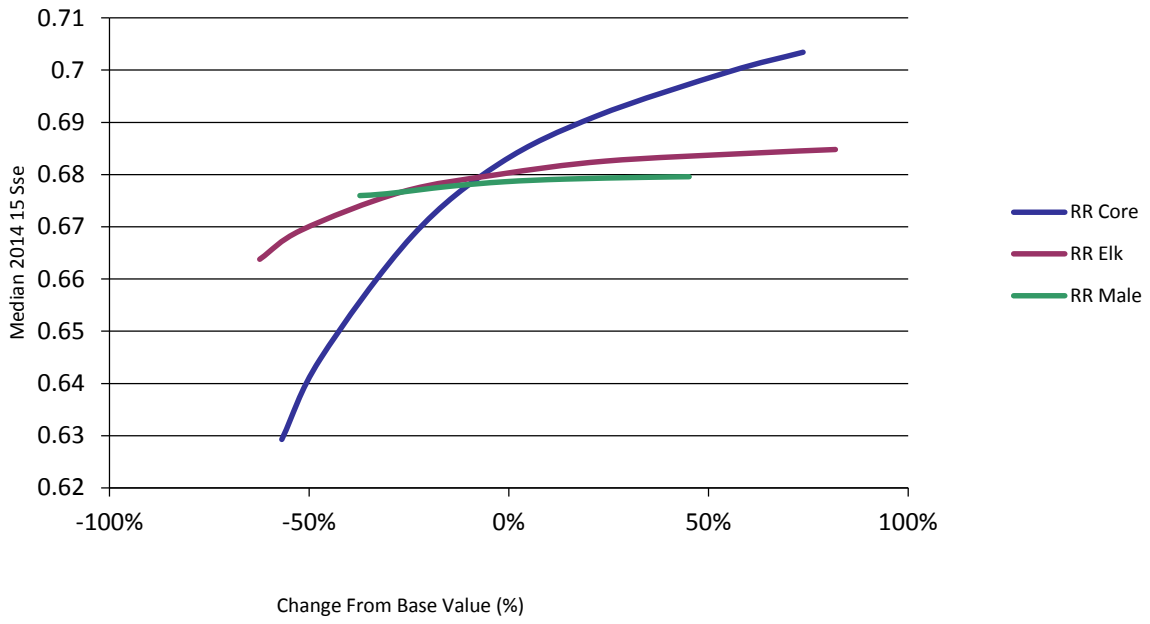

Figure 6.6 Change in median overall system sensitivity (2014 15) for three risk nodes included in wildlife scenario tree model.

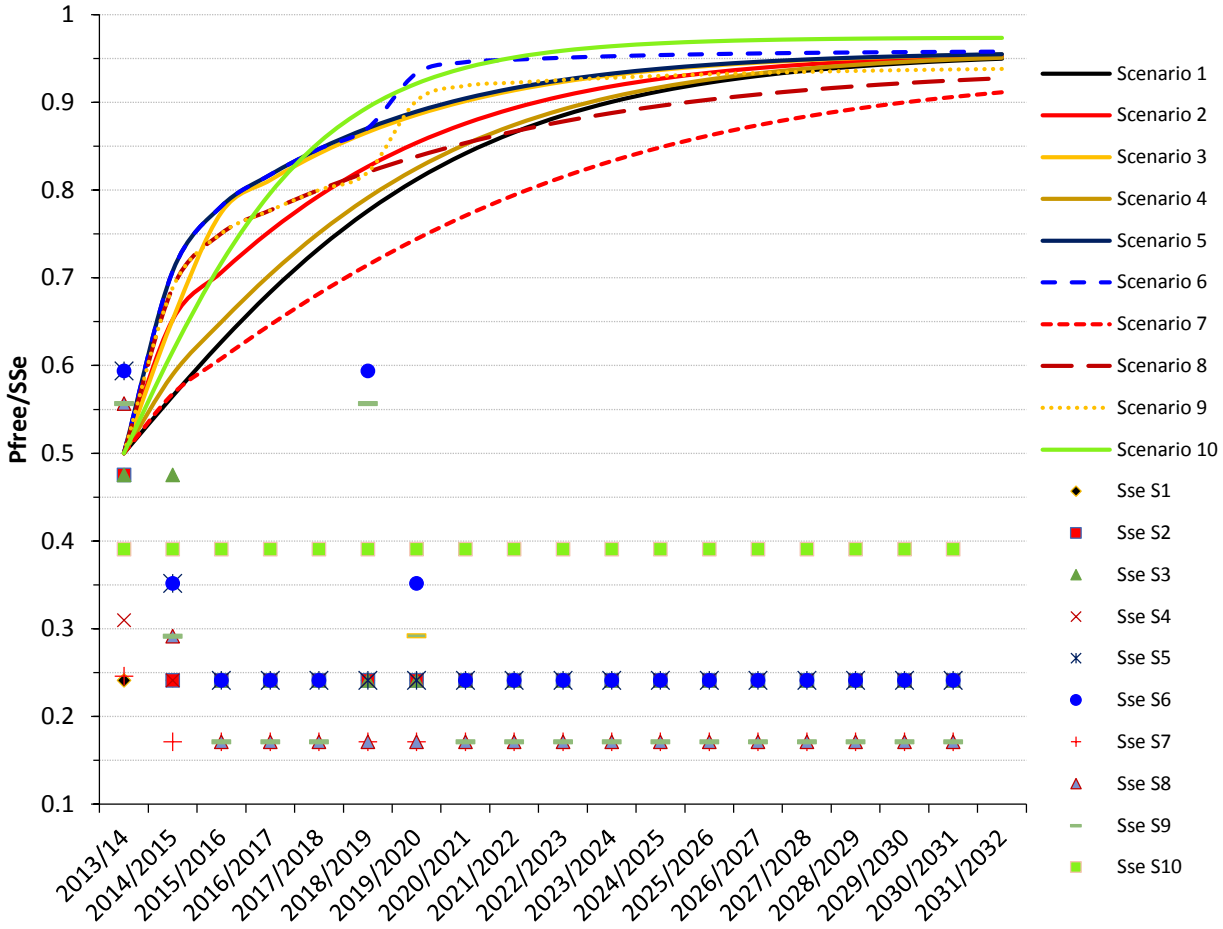

Figure 6.7 Probability of freedom (PFree) and overall system sensitivities (SSe) from 2013/14 to 2031/32 for ten future modeled scenarios.

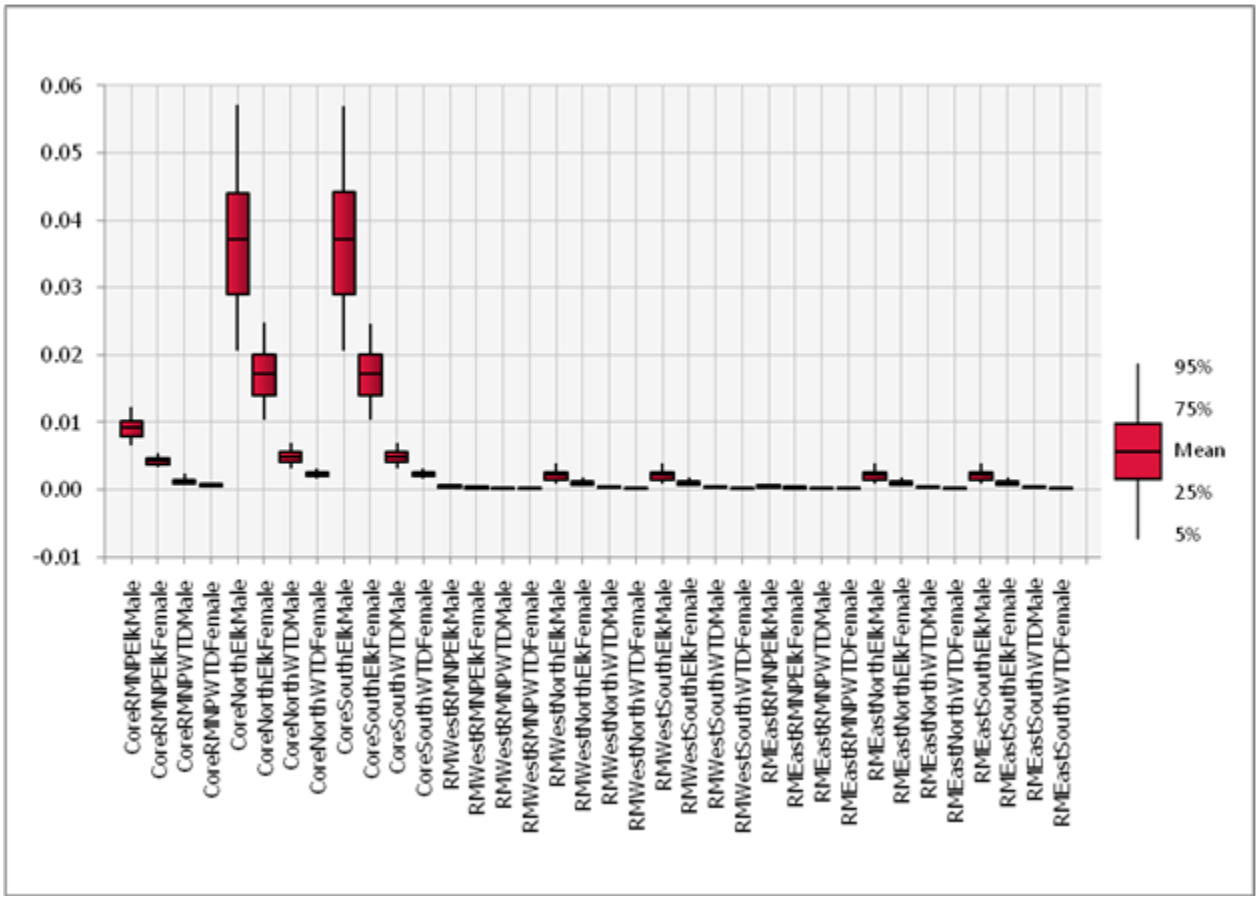

Figure 6.8 Box and whisker plot (mean +/- 95% CI) of effective probabilities of infection (EPI) for all combinations of risk category by subzone.

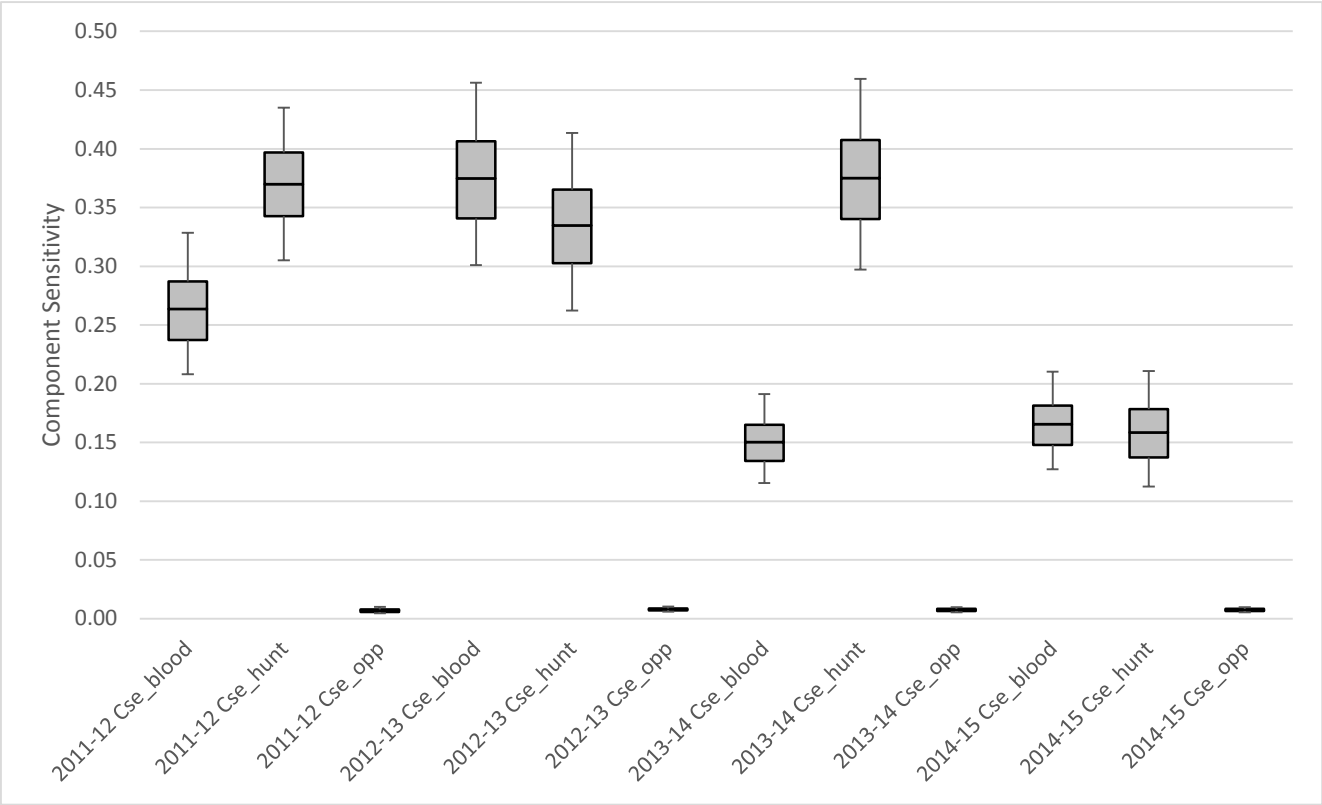

Figure 6.9 Component sensitivities for the blood test, hunt and opportunistic components of RMEA wildlife scenario tree from 2011-12 to 2014-15.

**Boxes represent 25th and 75th percentiles, line is mean and error bars represent 5% and 95% confidence intervals.

CHAPTER 7: CROSSING THE THRESHOLD: *MYCOBACTERIUM BOVIS* EPIDEMIOLOGY AND DISEASE MANAGEMENT IN THE GREATER RIDING MOUNTAN ECOSYSTEM

This chapter provides a synthesis of the previous chapters and attempts to tie the thesis together in a comprehensive way. This chapter also provides some 'lessons learned' from the management of M. bovis in this wildlife reservoir, one of the very few areas in the world where this disease has been successfully managed.

7.1 Introduction

The Greater Riding Mountain Ecosystem (GRME) is one of only four known wildlife reservoirs of *M. bovis* in North America and one of similar situations in a handful of countries dealing with such a reservoir globally (Fitzgerald and Kaneene, 2013). A similar, but functionally different wild cervid reservoir occurs in Michigan, USA where white-tailed deer (WTD) are the reservoir species and elk are a spillover host (O'Brien et al., 2006), while in Minnesota, a potential wildlife reservoir in WTD has recently been eliminated to below detectable levels (Carstensen and Doncarlos, 2011; Carstensen et al., 2011). The only other reservoir of *M. bovis* in North America occurs in wood bison in northern Canada, where it has existed since the mid 1920's and differs considerably in many aspects from the GRME (Nishi et al., 2006). *M. bovis* has likely been present within the GRME since at least 1978 and may have occurred there earlier, although undetected for many decades, in three wildlife species (elk, WTD, gray wolf), and one domestic species (cattle). This particular strain of *M. bovis* has never been detected in humans in Manitoba or other parts of Canada (pers. comm. M. Sharma, Public Health Agency of Canada). Several other potential wildlife reservoir species have been examined, but to date all have proven negative (bison (*Bison bison*), moose (*Alces alces*), beaver (*Castor canadensis*), coyote (*Canis latrans*), Richardson ground squirrel (*Urocitellus spermophilus*), fisher (*Martes pennanti*), raccoon (*Procyon lotor*), and pine

marten (*Martes americana*)). Elk were likely the primary reservoir species until recently with WTD, with cattle being spillover hosts (Lees, 2004; Shury and Bergeson, 2011).

Conceptually, wildlife reservoirs should be viewed within an ecosystem or landscape context which includes human and associated societal attitudes, something recently dubbed as an episystem by O'Connor et al (2012). I have found it useful to envisage the GRME as a disease-conducive landscape that favoured transmission and wildlife reservoir creation and maintenance in the late 1990's and early 2000's (Figure 7.1). Various combinations of host(s), agent, and environment working at multiple scales with 'environment' including human dynamics and sociocultural factors need to be considered as part of this episystem, as the social constraints of disease management often outweigh epidemiological considerations (i.e not all control methods will be accepted in all geographic areas)(Carter et al., 2009).

The following sections will attempt to summarize the current epidemiological knowledge of *M. bovis* in this ecosystem as well as the strategies used to manage the disease in the past two decades. I also provide a brief discussion of the lessons learned from management of this protracted outbreak, so that disease management agencies in future can learn and understand what factors are important for successful disease control. I believe there is firm evidence to use the term 'successful' for describing disease management efforts in the GRME as there have been no positive cases of *M. bovis* in cattle since 2008 (5 1/2 years as of this date), and the prevalence of infection in elk and WTD have dropped to levels that are very close to being undetectable at current surveillance levels (Chapter 4 & 6). This has been accomplished without the bitter and divisive media coverage and political controversy that has come to characterize a similar

problem in the United Kingdom where badgers are the primary wildlife reservoir and British society is divided on the use of badger culling to control bovine tuberculosis in cattle (Woodroffe et al., 2009; O'Connor et al., 2012; Munro, 2013). This study presents a unique opportunity to learn about the management, control and elimination of *M. bovis* from a wildlife reservoir both in a sociocultural context and an epidemiological context that will hopefully inform future efforts to manage disease outbreaks involving the wildlife-domestic animal-human interface in a 'One Health' approach.

7.2 Results and Discussion

The pathogenesis and lesion distribution of *M. bovis* in elk and WTD are very similar to other similar wildlife reservoirs (New Zealand, Michigan, Minnesota, Spain), with some notable exceptions (Chapter 2). The main difference is the proportion of non-visibly lesioned (NVL), culture positive animals that were found in this study compared to red deer, a closely related species in other countries where up to 30% of culture positive animals have no visible lesions (Lugton et al., 1998; Gavier-Widen et al., 2009). This could be due to differences between culture methods, necropsy protocols, disinfection protocols or strain differences.

One of the key aspects that led to control of this wildlife reservoir relatively quickly was the availability of accurate blood tests which allowed selective culling based on parallel interpretation of three blood tests from a single blood sample to quickly and accurately identify potentially infected elk and WTD (Chapter 3). The cost associated with this technique, was that a high proportion of animals that were false positive (positive on blood testing, negative on culture at necropsy), were culled. This was a preferred alternative to non-selective culling, as many more animals would have been removed using that method, so having a reliable screening test proved to be a key driver

of success, especially within RMNP, where hunting is not traditionally used as a wildlife control method. Reliance on a single test to diagnose *M. bovis,* especially in wildlife species, is likely not practical or wise and using multiple tests, either in parallel or series allows managers to tailor sensitivity and specificity to suit their specific needs within a specific context (Chapter 3). Wildlife managers should also utilize all opportunities under disease control programs to archive samples (especially sera, isolates and host DNA) to allow evaluation of newer diagnostic tests with well characterized banks of data. Selective culling was much more widely acceptable as a disease control measure than non-selective culling, as people were generally supportive of removing test positive animals rather than randomly selected animals. Considering the low prevalence of disease in this reservoir, this is quite understandable. If prevalence of disease were higher, then non-selective culling may be more acceptable, but acceptance of this method is a critical aspect for some key stakeholder groups that must be identified prior to undertaking major disease control activities. Therefore, any key stakeholder groups need to be meaningfully engaged early in a disease control program involving wildlife reservoirs.

The spatial distribution of wildlife cases and strains of *M. bovis* was quite restricted in this episystem and, despite the opportunity to spread for over 30 years, it has remained within a small area in the western portion of RMNP and surrounding area, with few exceptions (Chapter 5). Recent work suggests that WTD may be more likely than elk to spread *M. bovis,* due to their lack of population structure (Vander Wal et al., 2013a). Despite opportunity for spread, this does not seem to have occurred and other explanations should be considered. It is likely that WTD are a spillover host in this episystem and may not be agents of further geographic spread, as they do not maintain

latent infections for long periods of time because of their shorter life span compared to elk (Rue, 1997). Elk may be more important due to their higher degree of sociality which make them a better reservoir species and their longer lifespan, which may allow them to bridge the infection through time more efficiently than WTD. There is evidence from New Zealand that wild red deer may be playing this role in that episystem (Nugent, 2011).

The molecular and spatial epidemiology of *M. bovis* in the GRME suggest that a single, limited spillover event from cattle into wildlife occurred sometime in the past, but the source remains uncertain (Chapter 5). The strain of *M. bovis* found within the GRME is part of the EU1 clonal complex (Smith, 2012), so we can say with some certainty that the infection originally came from cattle and is not an 'ancestral' strain of *M. bovis* present in wildlife for several centuries. The most likely source is from co-grazing of cattle within RMNP which occurred between 1930 and 1970, with a less likely source being infected bison from BNP in the 1930's. The latter option is still a possibility until existing strain types are better characterized from the wood bison infected with *M. bovis* in northern Canada, where translocated bison from Buffalo National Park still reside (Chapter 1).

A combination of management factors initiated between 2000 and 2003, as part of the Manitoba Task Group for Bovine Tuberculosis has likely resulted in elk shifting from the major wildlife reservoir in this system to a spillover host, which is unable to maintain the infection at current densities (Chapter 4, 6). The vision of this multi-jurisdictional initiative was to eradicate bovine tuberculosis from the Riding Mountain ecosystem with three specific goals: 1) to achieve and maintain bovine bTB-free status in domestic cattle

, 2) to eradicate Bovine TB in wildlife that may pose a risk to agriculture, and 3) to minimize wildlife-livestock interactions in the Riding Mountain region, and to minimize unnatural cervid herding behaviour which occurs where cervids feed on agricultural products, thereby minimizing the potential for disease transmission (Tuberculosis, 2002). It could be argued that all three of these objectives are within reach in the forseeable future and some have been accomplished. Reduction in elk density was likely a key factor in the switch of elk from reservoir to spillover host, as evidenced by the strong positive association between elk density and odds of being culture positive in the logistic regression models in Chapter 4. WTD have likely always been a spillover host at densities at which they occur at within the GRME, and both cattle and WTD are likely spillover hosts in this episystem, but it is also possible that both are required to constitute a reservoir involving all three species. Disease transmission was reduced markedly as a result of efforts to prevent indirect contact between wild cervids and cattle over hay bales in winter, as there was a strong temporal association between reduced prevalence in wild cervids and fencing of hay storage yards around RMNP (Chapter 4).

M. bovis has likely been eliminated from the GRME or likely will be in the next five years, although there is very real potential for latent cases in elk and WTD in the coming decade. Results of the scenario tree model indicate that additional surveillance will be required, with a strong focus on the core area over at least a decade to ensure a probability of disease freedom above 95% (Chapter 6). While latent cases may still occur and be detected through surveillance, further transmission is unlikely, if conditions remain similar to present and densities are not allowed to increase and contact between cattle and wildlife is maintained. Current biosecurity practices which include

maintenance and use of barrier fencing for hay storage, use of guardian dogs, and strong legislation and enforcement of bans on baiting for hunting, will also need to be maintained. Ensuring adequate surveillance should be the focus of future management activities (Chapter 6), as the conditions are now in place that have allowed eradication to occur, much like has happened in other geographic locations in North America where sporadic *M. bovis* cases have occurred, and the disease apparently no longer exists (Belli, 1962; Friend et al., 1963; Steffen et al., 1999; Ryan et al., 2006b; Wobeser, 2009). These sporadic cases occurred while cattle populations still had a low prevalence of *M. bovis* and likely represented spillover into wildlife. White-tailed deer and elk densities where these isolated spillover events occurred were quite low and this is likely why these areas never developed into wildlife reservoirs (Wobeser, 2009). Cervid densities have generally increased throughout North America since the early part of the 20th century, concurrent with the development of wildlife reservoirs of *M. bovis* in several states and provinces (O'Brien et al., 2011a; Palmer et al., 2012). Free-ranging elk and WTD should be regarded as 'facultative' wildlife reservoir hosts, similar to hosts, such as ferrets and wild boar in other countries, as they can be considered as either a reservoir or spillover host for *M. bovis*, dependent primarily on their density and whether or not opportunities for close contact at local sources are abundant (i.e. baiting and feeding sites, congregation at waterholes in arid environments).

7.3 Conclusion

It is important to recognize that disease management objectives that have resulted in the success realized within the GRME were only accomplished through an ongoing adaptive management framework with constant integration of new knowledge and acceptance of various groups of engaged stakeholders. Of the management factors

instituted by the Manitoba TB Task Force, the ones that likely had the greatest impact in prevalence reduction in wildlife were fencing of hay storage yards around RMNP, selective culling of elk and WTD within RMNP, and density reduction of elk and WTD which appears to have reduced populations to below a critical community size (CCS) for maintenance of *M. bovis* in this wildlife reservoir. The concept of a threshold for disease maintenance has long been debated in the scientific literature (Lloyd-Smith et al., 2005; Potapov et al., 2012; Morters et al., 2013; Vander Wal et al., 2013b; Viana et al., 2014). I believe this study provides direct empirical evidence for the existence of thresholds in wildlife disease reservoirs, and is a primary example of epidemic fadeout following implementation of a suite of effective control measures. The implication is that *M. bovis* is transmitted primarily through a density dependent process, something for which some evidence exists, but other evidence suggests transmission is both frequency and density dependent (Vander Wal et al., 2013b).

The following section, although somewhat tangential to the objectives of this thesis as laid out in Chapter 1, are important to provide the social and policy context within which this study was carried out. All too often, these lessons are not written down and so wildlife managers often repeat the mistakes of history, instead of learning from others. Some of the lessons learned from disease management in the GRME can be summarized in six short statements, some of which are generalizable and others which are highly specific to the situation in southern Manitoba:

1) Minimal information about transmission and geographical extent of infection must be known before formulating and investing in disease management, so investment in basic research will pay dividends in the long run. In the GRME, significant efforts were made

to determine where infected elk and WTD were located in the first few years of surveillance.

2) The tail cannot be the only thing wagging the dog (impetus for disease management must be BOTH top down and bottom up in nature). Local cattle producers were very engaged early in the process resulting in high-level political involvement (agencies being asked to testify in front of the Federal Standing Committee on Agriculture), but interest and engagement waned over time as surveillance and research began to show results (no positive cattle cases). Governments must be open to collaborative decision making; something that does not come naturally to hierarchically organized bureaucracies.

3) Key stakeholder groups must be meaningfully engaged early in decision making and have a major stake in disease control objectives (they must benefit either directly or indirectly, otherwise they are not a true stakeholder). Finger pointing and standing on the sidelines and shouting at your neighbor to 'do something' is not productive. A concerted effort must be made to have the right people with open minds at the table that are willing to listen, and not just pontificate about their often unjustified, unmovable point of view.

4) A coordinated approach involving engaged stakeholders, various levels of government and aboriginal groups is required for successful wildlife disease management. Maintaining momentum with so many different actors is a major challenge, but having an independent third party act in a coordinator function helped re-engage agencies and stakeholders at a critical juncture. Communication is vital and major efforts to communicate new scientific findings as well as community concerns to decision makers must be undertaken on a continuous basis.

5) Wildlife disease management is inherently an adaptive process which is always shifting and changing, which makes it difficult for bureaucracies to keep up. New information must be continually sought out, both locally (by asking local communities what they think) and abroad (by finding out what worked elsewhere, and more importantly what doesn't work).

6) It takes a village to raise a child, but it takes a well-coordinated, transparent, slightly deranged, dedicated team of individuals to manage a wildlife disease outbreak.

In conclusion, the relatively successful management of *M. bovis* in the GRME has been the result of learning by doing. The disease situation was likely the result of spillover into wild cervids from domestic cattle, and resultant spillback into domestic cattle herds bordering Riding Mountain National Park several decades later. A suite of control measures instituted between 2000 and 2003 in the GRME, one of two remaining wildlife reservoirs of *M. bovis* in Canada, has resulted in a significant reduction in wild cervid prevalence over the past decade. Key control measures included comprehensive livestock testing, legislated and enforced bans on baiting and feeding of cervids, extended hunting seasons, fencing of winter hay storage yards, a moratorium on wolf hunting, and both selective and non-selective culling of elk and deer within Riding Mountain National Park. A single positive elk and no positive WTD have been detected in the past three years of surveillance (2011/12 to 2013/14, while the last infected cattle herd was detected in 2008. This study identified seven risk factors associated with infection in elk and deer. The probability of an animal testing positive was positively associated with sex, age category, geographic area, and elk density and negatively associated with year category.

MIRU-VNTR and spoligotyping were used to characterize the spatial distribution of isolated obtained from wildlife and cattle since 1990, demonstrating only two spoligotypes and two MIRU-VNTR types associated with elk, cattle and deer. This is likely a clonal expansion due to limited spillover that has persisted for at least 35 years and appears to be eliminated, or is at least below the threshold where ongoing transmission can occur. Elk density reduction, baiting bans, natural predation, selective culling, along with measures to reduce wildlife-cattle interaction in winter, were key factors leading to relatively rapid control of this wildlife reservoir. Wild cervid populations should be regarded as facultative reservoir hosts of *M. bovis*, dependent on risk factors including host density and presence of baiting and feeding. A density threshold or critical community size (CCS) of approximately 1 elk per square kilometre seems to exist within the elk population of this system, below which *M. bovis* cannot be sustained. This threshold is likely not precisely defined and caution should be exercised when extrapolating this to other episystems, as local circumstances will likely have significant effects.

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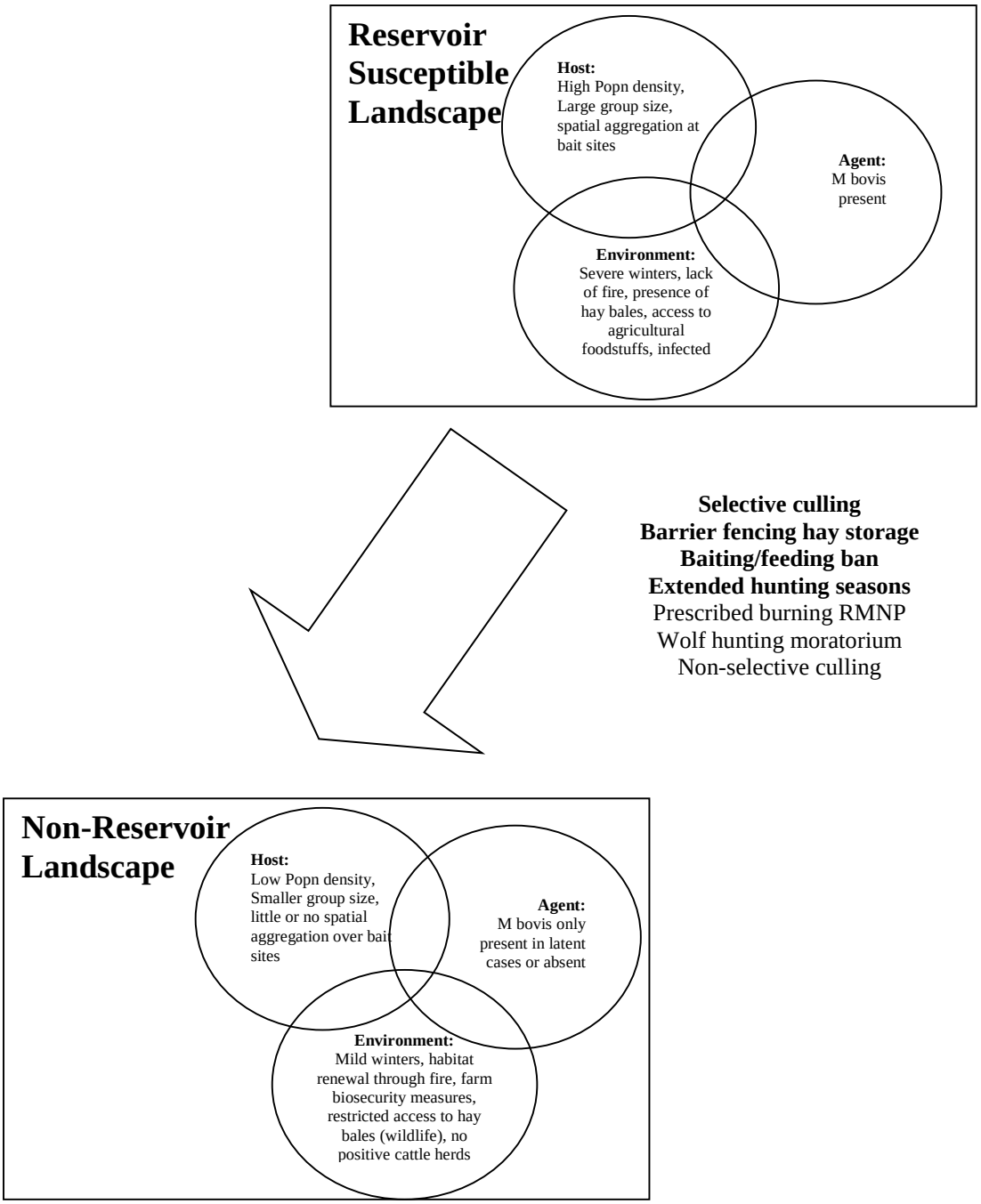


Figure 7.1 Conceptual representation of the 'reservoir landscape' in the GRME and how that has changed over time.

APPENDIX

KERNEL DENSITY VALUES AND NODE STATISTICS FOR BAYESIAN ANALYSIS OF ELK AND WHITE-TAILED DEER BLOOD TEST VALIDATION

1) Parameters for Elk

Conditional dependence model (Bayesian latent class model)

Node statistics

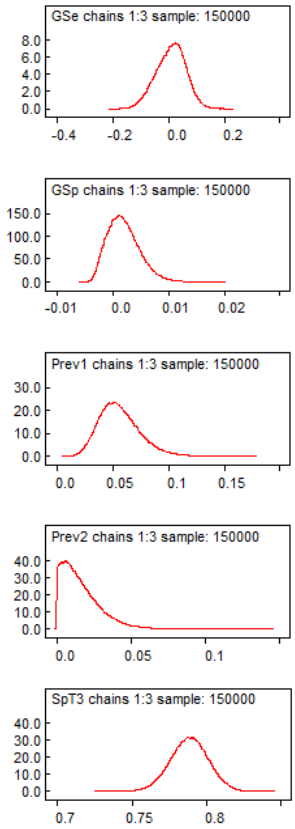
node	mean	sd	MC error	2.5%	median	97.5%	start	sample
GSe	0.008848	0.0527	2.023E-4	-0.09847	0.01197	0.106	5001	150000
GSp	0.001959	0.002824	9.815E-6	-0.002606	0.001678	0.008236	5001	150000
Prev1	0.05624	0.01815	9.353E-5	0.02647	0.05423	0.0975	5001	150000
Prev2	0.01655	0.01339	6.205E-5	6.775E-4	0.01341	0.05038	5001	150000
SeT1	0.3783	0.1076	4.958E-4	0.1932	0.3699	0.6105	5001	150000
SeT2	0.7558	0.08907	3.898E-4	0.5623	0.7636	0.9058	5001	150000
SeT3	0.7103	0.07547	3.346E-4	0.5518	0.7144	0.8454	5001	150000
SpT1	0.9354	0.008491	2.594E-5	0.9179	0.9356	0.9513	5001	150000
SpT2	0.9441	0.009848	4.793E-5	0.9254	0.9439	0.9641	5001	150000
SpT3	0.7886	0.0127	3.781E-5	0.7633	0.7887	0.8132	5001	150000

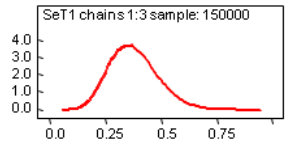
DIC

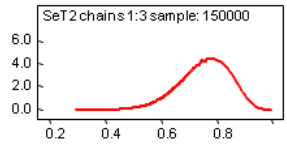
Dbar = post.mean of -2logL; Dhat = -2LogL at post.mean of stochastic nodes

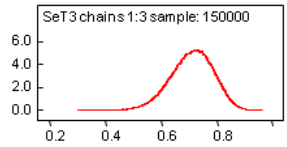
	Dbar	Dhat	pD	DIC
y1	47.658	44.505	3.153	50.811
y2	42.576	40.656	1.919	44.495
total	90.234	85.161	5.072	95.306

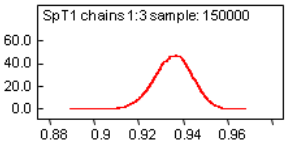
Kernel density

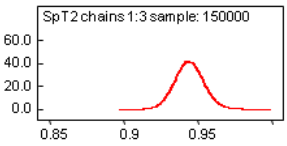

GSe chains 1:3 sample: 150000
8.0
6.0
4.0
2.0
0.0
-0.4
-0.2
0.0
0.2
GSp chains 1:3 sample: 150000
150.0
100.0
50.0
0.0
-0.01
0.0
0.01
0.02
Prev1 chains 1:3 sample: 150000
30.0
20.0
10.0
0.0
0.0
0.05
0.1
0.15
Prev2 chains 1:3 sample: 150000
40.0
30.0
20.0
10.0
0.0
0.0
0.05
0.1
SpT3 chains 1:3 sample: 150000
40.0
30.0
20.0
10.0
0.0
0.7
0.75
0.8











Conditional independence (CID) model (Bayesian latent class analysis)

Node statistics

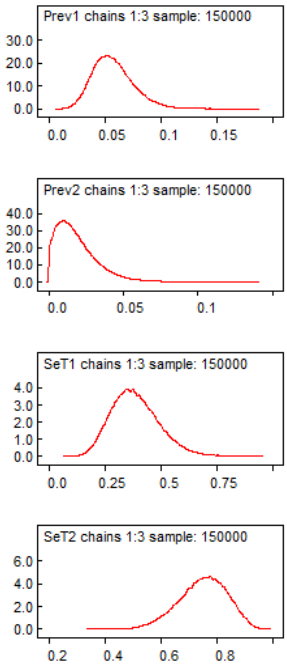
node	mean	sd	MC error	2.5%	median	97.5%	start	sample
Prev1	0.05764	0.01818	9.021E-5	0.02762	0.05571	0.09855	5001	150000
Prev2	0.01857	0.01358	5.988E-5	0.001184	0.01572	0.05206	5001	150000
SeT1	0.3875	0.1058	4.399E-4	0.2086	0.3782	0.6189	5001	150000
SeT2	0.7512	0.08626	2.862E-4	0.5678	0.7572	0.9015	5001	150000
SeT3	0.7045	0.07438	3.09E-4	0.5491	0.7082	0.8384	5001	150000
SpT1	0.9369	0.008193	2.263E-5	0.9201	0.9372	0.9522	5001	150000
SpT2	0.9452	0.009541	4.534E-5	0.927	0.945	0.9647	5001	150000
SpT3	0.7888	0.0126	3.659E-5	0.7636	0.7889	0.8132	5001	150000

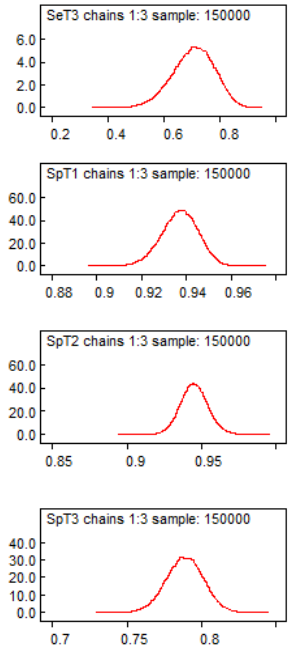
DIC

Dbar = post.mean of -2logL; Dhat = -2LogL at post.mean of stochastic nodes

	Dbar	Dhat	pD	DIC
y1	46.550	43.671	2.878	49.428
y2	43.709	42.106	1.602	45.311
total	90.258	85.778	4.481	94.739

Kernel density


Prev1 chains 1:3 sample: 150000
30.0
20.0
10.0
0.0
0.0
0.05
0.1
0.15
Prev2 chains 1:3 sample: 150000
40.0
30.0
20.0
10.0
0.0
0.0
0.05
0.1
SeT1 chains 1:3 sample: 150000
4.0
3.0
2.0
1.0
0.0
0.0
0.25
0.5
0.75
SeT2 chains 1:3 sample: 150000
6.0
4.0
2.0
0.0
0.2
0.4
0.6
0.8


SeT3 chains 1:3 sample: 150000
6.0
4.0
2.0
0.0
0.2
0.4
0.6
0.8
SpT1 chains 1:3 sample: 150000
60.0
40.0
20.0
0.0
0.88
0.9
0.92
0.94
0.96
SpT2 chains 1:3 sample: 150000
60.0
40.0
20.0
0.0
0.85
0.9
0.95
SpT3 chains 1:3 sample: 150000
40.0
30.0
20.0
10.0
0.0
0.7
0.75
0.8

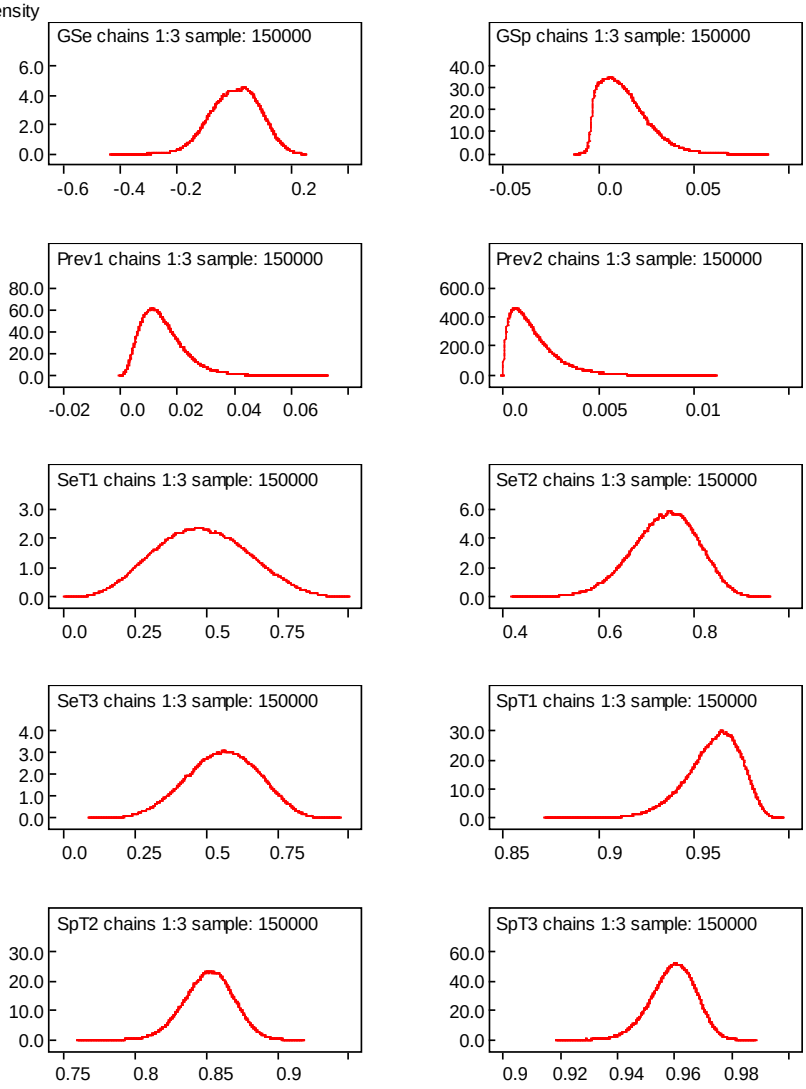
2) Parameters for White-tailed Deer

Conditional dependence model for WTD (Bayesian latent class model)

Node statistics

node	mean	sd	MC error	2.5%	median	97.5%	start	sample
GSe	0.001754	0.08325	2.731E-4	-0.1657	0.005012	0.1532	5001	150000
GSp	0.0133	0.01205	5.923E-5	-0.003347	0.01132	0.04156	5001	150000
Prev1	0.01468	0.007412	2.408E-5	0.0039	0.01345	0.03238	5001	150000
Prev2	0.001575	0.001211	4.466E-6	1.416E-4	0.001278	0.004683	5001	150000
SeT1	0.4853	0.1585	5.353E-4	0.1913	0.4817	0.7964	5001	150000
SeT2	0.7386	0.06883	2.45E-4	0.5939	0.7427	0.8611	5001	150000
SeT3	0.5583	0.1251	3.157E-4	0.3081	0.5614	0.7901	5001	150000
SpT1	0.9599	0.01447	7.499E-5	0.9271	0.9617	0.983	5001	150000
SpT2	0.8517	0.01707	4.65E-5	0.8169	0.8522	0.8837	5001	150000
SpT3	0.9594	0.007779	2.157E-5	0.9429	0.9598	0.9733	5001	150000

Kernel density



Conditional independence model for WTD (Bayesian latent class model)

Node statistics

node	mean	sd	MC error	2.5%	median	97.5%	start	sample
Prev1	0.0126	0.006394	2.069E-5	0.003343	0.01154	0.02789	5001	150000
Prev2	0.00152	0.001172	4.638E-6	1.338E-4	0.001231	0.004501	5001	150000
SeT1	0.5541	0.1598	3.974E-4	0.2397	0.5581	0.8465	5001	150000
SeT2	0.5168	0.1272	3.333E-4	0.2716	0.5172	0.7614	5001	150000
SeT3	0.7415	0.06828	1.774E-4	0.5978	0.7455	0.8632	5001	150000
SpT1	0.911	0.01783	4.954E-5	0.8731	0.9119	0.9429	5001	150000
SpT2	0.9798	0.005383	1.48E-5	0.9681	0.9803	0.989	5001	150000
SpT3	0.856	0.01693	4.352E-5	0.8215	0.8566	0.8877	5001	150000

Kernel density